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A role for interleukin-1β in synaptic function.

By

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Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College.

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I declare that this thesis is entirely my own work with the following exceptions: the docosahexnaoic diet on young and aged rats referred to in chapter 5 was organized by Dr. B. McGahon; all of the LTP experiments which were carried out by Prof. Marina Lynch. This work has not been previously submitted as an exercise for a degree to this or any other University. I give permission to the library to lend or copy this thesis .

Emily Verdeck

V List of abbreviations

AA	Arachidonic Acid
ACPD	1-amino-cyclopentane-1,3-dicarboxylate
AD	Alzheimer's disease
AFT-2	Activating transcription factor-2
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ATP	Adenosine 5'-triphosphate
BaP	Benzo(a)pyrene
β-APP	β-amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaM kinase II	Calcium/calmodulin kinase II
CAT	Catalase
CNS	Central nervous system
CO	Carbon monoxide
CSF	Cerebrospinal fluid
CVO	circumventricular organs
DAG	Diacylglycerol
DCF	2'7'-dichlorofluorscein
DCFH DA	2'7'-dichloroflourscin diacetate
DHA	Docosahexanoic acid
DMSO	Dimethyl sulphoxide
EAA	Excitatory amino acids
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethylether) *N,N* 'tetraacetic acid
EPSP	Excitatory postsynaptic potential
ERK	Extra-cellular signal regulated kinase
FGF	Fibroblast growth factor
FIL	Family of IL-1
GABA	γ-aminobutyrate
G-Protein	GTP-binding protein
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GTP	Guanosine 5'-diphosphate
H_2O_2	Hydrogen peroxide
HFS	High frequency stimulation
ICE	Interleukin-1β-converting enzyme
IFN-γ	Interferon gamma
IGIF	Interferon gamma-inducing factor
IKK	I-kappa B kinases
IL	Interleukin
IL-1α	Interleukin-1α
IL-1β	Interleukin1-1β
IL-1ra	Interleukin-1 receptor antagonist
IL-1RI	Interleukin-1 type I receptor
IL-1RII	Interleukin-1 type II receptor

IL-1RAcP	Interleukin-1 receptor accessory protein
IRAK	Interleukin-1RI- receptor associated kinase
IL-1RrP	Interleukin-1 receptor-related protein
JNK	c-jun N-terminal kinase
KCl	Potassium chloride
LPS	Lipopolysaccharide
LBP	lipopolysaccharide-binding protein
LTP	Long-term potentiation
MAP kinase	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NIK	NF-κB-inducing kinase
NK	Natural killer
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
PAF	Platelet activating factor
PD	Parkinson's disease
PDGF	Platelet derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear
PMSF	Phenylmethylsulphonyl fluoride
PUFA	Polyunsatyurated fatty acid
rIL-1	recombinant IL-1
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TLR	Toll like receptor
TMB	3,3',5,5'-Tetramethyl-benzidine
TMT	Trimethyltin
TNF-α	Tumour necrosis factor-α
TNFRI	p55/TNF receptor type I
TRAF	TNF receptor-associated family
trk	Receptor tyrosine kinase
TUNEL	TdT-mediated dUTP Nick-End-Labelling

V Abstract

The proinflammatory cytokine, interleukin-1β (IL-1β), one of the most extensively studied cytokines to date, is known to play a pivotal role in neurodegenerative, inflammatory and infectious diseases. Moreover, IL-1β has been implicated in the impairment of long-term potentiation (LTP), a model for learning and memory, and at least one biochemical correlate associated with LTP, i.e., glutamate release. The mechanisms underlying the impairment of LTP in deleterious circumstances, such as in ageing, remains to be fully elucidated. The focus of this study involved investigating the possibility that the stress-activated protein kinases, JNK and p38, underlie this impairment and that activation is initiated by an increase in IL-1β concentration and reactive oxygen species (ROS) formation.

This study shows that IL-1β induces an increase in the activity of JNK and p38, coupled with an inhibition of glutamate release in both hippocampal and dentate gyrus synaptosomes *in vitro*. These data suggest that p38 and JNK play a possible role in the IL-1β-induced inhibition of glutamate release. This theory is supported by the finding that the p38 inhibitor, SB 203580, reverses the inhibitory effect of IL-1β on glutamate release *in vitro*.

The effect of intracerebroventricular injection of IL-1β on LTP in the rat dentate gyrus was also assessed. The data showed that IL-1β injection impaired LTP and glutamate release and both effects were coupled with increases in ROS formation and JNK and p38 activity. It is proposed, therefore, that ROS play a role in the IL-1β-induced impairment in LTP, glutamate release and increase in JNK and p38 activity. To further investigate this proposal, an anti-oxidant diet, enriched in vitamin E and vitamin C, was fed to rats pretreated with IL-1β. The results showed that the antioxidant diet reversed the IL-1β-induced effects.

Aged rats are unable to sustain LTP, and there is some evidence to suggest that this effect is associated with an increase in IL-1β concentration and ROS formation. This study investigated this possibility and found that, coupled with the increase in IL-1β concentration, was an increase in the activity of p38 and JNK. In aged rats fed on a diet enriched with the polyunsaturated fatty acid, docosahexanoic acid, the age-related impairment in LTP as well as the age-related increases in IL-1β, JNK and p38 were reversed.

It has been shown that lipopolysaccharide (LPS), derived from the cell wall of Gram-negative bacteria, increased IL-1β concentration in the brain. To further establish the negative correlation between IL-1β and LTP, LPS was used as an experimental tool. LPS was shown to impair the expression of LTP and glutamate release and these changes were accompanied by an increase in the activity of the IL-1β-converting enzyme (ICE), IL-1β concentration, ROS formation, JNK and p38 activity. It was also shown that the ICE inhibitor, Ac-YVAD-CMK, reversed the LPS-induced impairment of LTP, suggesting that the effects of LPS were mediated by IL-1β and suggesting that ICE is necessary for the activation of IL-1β. Associated with LPS, was an increase in (1) NF-κB activity, (2) apoptotic hippocampal and entorhinal cortical cell death and (3) caspase-3 activity; the latter two effects being markers of cell death. It is, therefore, hypothesized that the LPS-induced impairment in the expression of LTP may be partly due to a decrease in the viability of the cells of the entorhinal cortex and dentate gyrus.

These data functionally link p38 and JNK to impaired synaptic function in circumstances following treatment with IL-1β or LPS, or in ageing and suggest that JNK and p38 negatively impact on the expression of LTP by downregulating glutamate release and/or by decreasing cell viability.

Chapter 1

General introduction

1.1 The Interleukin-1 family

1.1.1 IL-1α and IL-1β

The proinflammatory cytokine interleukin-1 (IL-1) consists of three hormone-like polypeptide proteins that play an pivotal role in the mediation of the host response to systemic and local injury (Loddick *et al.*, 1998, Schneider *et al.*, 1998; see Rothwell, 1999). The members of this family include two agonists, IL-1α and IL-1β and an IL-1 receptor antagonist (IL-1ra), which has the capacity to inhibit the actions of IL-1α and IL-1β. These proteins share 19-26 % sequence homology, but are derived from different gene products (see Feghali & Wright, 1997). Another member of the IL-1 family has also been described within the past few years and was formerly called interferon-gamma inducing factor (IGIF), but has subsequently been renamed IL-18. IL-18 displays similar functional qualities to that of other members in the IL-1family, such as receptor expression, processing, and regulatory functions (Lebel-Binay *et al.*, 2000)

However it has recently been discovered that four additional proteins show similarities to the IL-1 family. These proteins have been cloned and shown to have similar sequence homology and protein structure to IL-1α, IL-1β, IL-1ra and IL-18. These four new members have been called FIL delta, FIL epsilon, FIL zeta and FIL eta, whereby FIL stands for "family of IL-1" (Smith *et al.*, 2000). The intron-exon organization of the extended interleukin-1 family suggests that the family originated from a common ancestral gene 350 million years ago (see Dinarello, 1996). Mature IL-1α and IL-1β have molecular weights of about 17 kDa. IL-1β has been shown to be the predominant form of IL-1 released from cells in the brain and elsewhere, whereas IL-1α is released to a lesser extent and slightly later (Ayala *et al.*, 1994; see Rothwell, 1999).

IL-1α and IL-1β exist as precursors, each of 31 KDa that lack leader sequences. Pro-IL-1α is biologically active, but remains predominantly in the cell. However, when cell death occurs, pro-IL-1β is released and is capable of being cleaved by extracellular proteases to give mature IL-1β (see Dinarello, 1996). However, for IL-1β to become biologically active, it must be cleaved by a specific cellular protease and the processing of pro-IL-1β (31kDa) to the mature form requires an important cleavage step. The IL-1β-converting enzyme (ICE), caspase-1, is a specific monocytic cell cytoplasmic cysteine protease which proteolytically cleaves

pro-IL-1β to its mature and active 17 kDa form (Thornberry *et al.*, 1992; Walker *et al.*, 1994; Ayala *et al.*, 1994). The mechanism by which IL-1β exerts its actions is receptor-mediated, which will be discussed in section 1.1.4.

Numerous studies have addressed the precise location and cellular source of IL-1 and IL-1mRNA in the periphery and central nervous system. Peripheral immune cells such as macrophages and neutrophils, are a common source of cytokines. Experimental models that exhibit excitotoxic, traumatic and ischemic damage have been used to elucidate the precise location of IL-1 activity. Additionally, systemic kainic acid administration is a commonly used tool to induce convulsions and seizures. These experimental models induce brain neurodegeneration and trauma, which provide excellent methods with which to study the central biological activities of IL-1 (Errikson *et al.*, 1999). The NMDA-receptor agonist, cis-2,4-methanoglutamate (MGlu), induces acute excitotoxicity, which consequently activates IL-1β production in the rat cortex and striatum by microglia and astrocytes (Pearson *et al.*, 1999).

Immunohistochemical studies have shown that, 5 hours (h) after kainic acid administration, IL-1β immunoreactive cells were visible in the hippocampus, thalamus, amygdala, piriform, perihinal cortex and hypothalamus. After a 12h exposure, the reactive cell number was increased. At 24 h, IL-1β-labelled cells were visible in the hippocampus and on the pyramidal cell layer of the CA1 and CA3 fields of the hippocampus. Immunoreactivity of IL-1β was associated predominantly with microglial cells (Eriksson *et al.*, 1999). Another study demonstrated that focal intrahippocampal application of kainic acid resulted in an early induction of IL-1β in parallel with up-regulation of IL-1β mRNA. In that situation IL-1β immunoreactivity was increased in glial cells, corresponding to activated microglia. Although no neuronal loss was evident, it is thought that degenerating neurones may be involved in some signal transduction mechanism to induce IL-1β activation (Eriksson *et al.*, 1999). Reportedly, microglial cells are rapidly activated upon pathological changes in the central nervous system (Vezzani *et al.*, 1999). Intense exposure of mice to trimethyltin (TMT), a hippocampal convulsant which induces learning and memory deficits, aggression and hyperactivity, has been also used as an experimental tool to establish localization of IL-1 mRNA following stressful stimuli. At various time points following intraperitoneal injection of TMT, mRNA levels for IL-1α and IL-1β

were increased, in parallel with an increase in microglial cell infiltration into the hippocampus (Bruccoleri et al., 1998)

1.1.2 The interleukin-1 receptor antagonist (IL-1ra)

The antagonist member of the IL-1 family is the IL-1 receptor antagonist (IL-1ra). This polypeptide was initially discovered as an IL-1 inhibitor in the urine of patients with fever (Arend *et al.*, 1993). In 1990, the complementary DNA (cDNA) for IL-1ra was cloned and expressed in *E. Coli.* (Eisenberg *et al.*, 1990). It has been established that IL-1ra shares 20-25% homology with IL-1α and IL-1β (see Feghali & Wright, 1997). IL-1ra is thought to be the only known endogenous receptor antagonist that is selective and specific for blocking the actions of IL-1α and IL-1β. IL-1ra has two structural variants, firstly a 17kDa form, known as secretory IL-1ra (sIL-1ra), derived from macrophages, monocytes, and neutrophils. The second variant is an 18kDa intracellular form of IL-1ra (icIL-1ra), which remains within the cytoplasm of keratinocytes, fibroblasts, and monocytes. The sIL-1ra form is produced by almost any cell that has the capability of producing IL-1, with the possible exception of hepatocytes and endothelial cells (Arend *et al.*, 1998).

IL-1ra acts functionally by binding to both type I and type II receptors (IL-1RI and IL-1RII respectively), but preferentially to the type I receptor (Sims *et al.*, 1994). IL-1ra does not preferentially block either IL-1α and IL-1β, as it has the capability to block both forms of IL-1 (Rothwell *et al.*, 1997). The binding of IL-1ra to the IL-1β complex different from that in other cytokine-receptor complexes, which maybe due to the structural variations found between cytokine-receptor complexes (Schreuder *et al.*, 1997).

To date, there are no reports suggesting that IL-1ra exhibit any agonist activities. Many experimental tools have been employed in an attempt to elucidate the precise mechanism by which IL-1ra works and also to establish the localization of IL-1ra in the central and peripheral nervous system. Recombinant IL-1ra has been used experimentally *in vivo* and *in vitro* and has been shown to eliminate or inhibit most known activities of IL-1. For example, intracerebroventricular (i.c.v.) injection of IL-1ra reduces damage induced by focal, global, permanent or reversible ischemia, excitotoxic, traumatic or inflammatory brain damage (Relton *et al.*, 1996). Many studies have examined the role of endogenous IL-1ra in various conditions and

systems in animal models and human diseases. Increased concentrations of IL-1ra have been reported in synovial fluid from patients with conditions such as rheumatoid and chronic arthritis; results suggest that the predominant source of IL-1ra in these circumstances is synovial macrophages (Arend *et al.*, 1998). *In vitro* studies have demonstrated that large quantities of IL-1ra must be administered due, to some extent, to the short half-life of IL-1ra, which is 6 minutes. An example of this has been shown in cases where exogenous IL-1 has been injected intravenously, followed by a 100-1000 fold excess of IL-1ra in order to prevent the biological activity of IL-1 agonists (Arend *et al.,* 1990). This excess administration of IL-1ra is also necessary due to an abundance of 'spare' IL-1 receptor sites for which IL-1ra must compete. It may also be the case that biologically inactive IL-1 type II receptor may compete for the available IL-1ra (Ruggiero *et al.*, 1997).

Many clinical trials have been carried out to examine the possibility of employing IL-1ra in the treatment of various diseases. This treatment was considered in patients suffering for rheumatiod arthritis, osteoarthritis, sepsis syndrome, asthma, graft-versus-host disease and inflammatory bowel disease. However reports show that the local delivery of IL-1ra through gene-therapy may be more effective in rheumatic diseases than systemic administration of IL-1ra (Arend *et al.*, 1998)

1.1.3 Interleukin-18 (IL-18)

Another proposed member of the IL-1 superfamily is IL-18, also known as interferon gamma-inducing factor (IGIF). IL-18 shares structural homology with IL-1β and IL-1ra (Prinz & Hanisch, 1999). This 18 kDa proinflammatory cytokine was first described in 1989 and subsequently cloned from mouse liver during toxic shock (Nakamura *et al.,* 1989, Okamura *et al.,* 1995). IL-18 is secreted primarily from macrophages and Kupffer cells and is primarily associated with the induction of IFN-γ production from $CD4^+$ T_h1 cells, B cells and natural killer (NK) cells in the presence of IL-12 (Tominaga *et al.*, 2000). Monocytes, macrophages and keratinocytes have been shown to be a major source of IL-18. Furthermore, osteoclasts and keratinocytes have been shown to be an abundant source of IL-18, suggesting similar functional qualities to that of IL-1β, which is involved in the regulation of bone density (Dinarello, 1999). IL-18 and IL-1β share similar characteristics, thus IL-18 is more related to IL-1β than any other cytokine.

Structurally both cytokines are β-sheet-folded peptides, which is of significant biological importance since very few cytokines have this structural feature (see Dinarello, 1999).

Despite the similarities between IL-1β and IL-18, they display different biological actions expressed through their specific receptors. IL-18 has been reported to act on T_h1 cells but not T_h2 cells to enhance IFN-γ production. The biological activities of IL-18 and IL-1β show that, in terms of enhancing IFN-γ production, NK cell toxicity and granulocyte-macrophage colony-stimulated factor production, IL-1β is associated primarily with the activation of T_h2 cells, unlike IL-18 which is associated with T_h1 cell activation (Akita *et al.*, 1997). IL-18 is processed in a manner similar to that of IL-1β, whereby it must be cleaved from the pro-IL-18 form to a mature form to become biologically active. Like IL-1β, IL-18 is also a substrate for ICE and is, in fact, thought to be the preferred substrate for this enzyme (Rano *et al.*, 1997). However it has also been shown that another caspase, CPP32/caspase-3 is involved in IL-18 activation. Endogenous CPP32/caspase-3 has been shown to process human pro-IL-18 to a biologically inactive form. It has been proposed that both ICE and CPP32/caspase-3 can act in concert to either activate or inactivate a switch for IL-18 (Akita *et al.*, 1997). Toxins from Gram-negative and Gram-positive bacteria activate macrophages to IL-18. There is little evidence to suggest the precise cell type-specific synthesis and the biological activities of IL-18 in the central nervous system. However, it has been show that IL-18 exerts its actions in the murine brain and in primary glial cell cultures (Prinz & Hanisch, 1999).

1.1.4 Receptor activation

The mechanism by which IL-1α and IL-1β exert their actions is receptor mediated. Two receptors have been identified to date, type I (80 kDa) receptor (IL-1RI) and type II (68 kDa) receptor (IL-1RII). IL-1RI has the ability to bind to both IL-1α and IL-1β in a similar manner and is considered to be the active IL-1 receptor (Rothwell & Strijbos, 1995; Hammond *et al.*, 1999). These two receptors bind all three members of the IL-1 family with varying affinities. IL-1RI binds all three ligands with similar affinity, whereas IL-1RII has a stronger binding affinity for IL-1β rather than IL-1α (Loddick *et al.*, 1998). In order for IL-1 to trigger intracellular

signalling by binding to the type I receptor, an accessory protein (AcP) is required for IL-1 signal transduction and has been called the IL-1 receptor accessory protein (IL-1R AcP). When IL-1 binds to IL-1RI, forming a complex, IL-IR AcP then binds to this complex, triggering signal transduction (Greenfeder *et al.,* 1995). It has been established that this complex is essential for signalling, whereby cells deficient in IL-1R AcP are unable to respond to IL-1. It has also been shown that IL-1R AcP is essential for the activation of IL-1RI- associated protein kinase (IRAK), and the stress-activated kinases (Wesche *et al.,* 1997). It has also been determined that IL-1R AcP is essential for the IL-1-induced activation of NFκB (Hofmeister *et al.*, 1997). IRAK and cell signalling will be discussed later in more detail in section 1.6.5. There has been much speculation about the presence of other IL-1 receptors and reports show that a recently cloned IL-1 receptor-related protein (IL-1RrP) shares homology with IL-1RI and IL-1RII. However despite the similarities between IL-1RrP and the other receptors, it is probably not an IL-1 receptor as it has no capability to bind other IL-1 ligands. The three proteins identified to date which share homology with IL-1RI, are T1/ST2, IL-1Rrp and IL-1AcP (Parnet *et al.,* 1996).

It is thought that the type II receptor does not mediate IL-1 signal transduction. IL-1RII is expressed as a membrane bound protein (68 kDa) or as a soluble receptor (45 kDa). When shed from the membrane in response to various extracellular stimuli, the soluble form acts as a decoy by binding IL-1. It has been proposed that this "decoy" receptor may play a role in regulating circulating levels of IL-1β (Hammond *et al.,* 1999). Furthermore, the membrane–bound type II receptor associates with the AcP to reduce the number of IL-1RI/AcP complexes formed (Malinowsky *et al.,* 1998).

Much evidence has been accumulated about the relative distribution IL-1 binding sites and mRNA for IL-1 receptors by performing autoradiographic studies, *in situ* hybridization studies and RNase protection studies in both murine and rat models. RNase protection studies show that mRNA for IL-1R1 is detectable in rat brain. The membrane–bound form of IL-1R1, however, was detectable in brain regions such as the thalamus, neostriatum, hippocampus, medulla, midbrain and cerebral cortex. In parallel with these findings, it was also established that mRNA for IL-1RAcP was expressed abundantly in the hippocampus, primarily in the dentate gyrus, but not within the neighbouring CA fields. Additional evidence showed that

IL-1R1 and IL-1RAcP mRNA was apparent in non-neuronal cells, such as blood vessels and parenchymal cells (Loddick *et al.*, 1998).

IL-18 acts by activating a specific receptor. The IL-18 receptor (IL-18R) complex consists of IL-1R-related protein (IL-18Rα) and IL-1R accessory protein-like protein (IL-18Rβ), both of which belong to the IL-1R family. The IL-18R partly shares the signalling pathway of IL-1R, suggesting that the IL-18-IL-18R complex is similar to the IL-1-IL-1R complex (see Dinarello, 1999; Tominaga *et al.*, 2000). The human IL-18 receptor (hIL-18R) was first purified and characterized from a Hodgkin's disease-derived cell line, known as L428, which express hIL-18R in abundance at approximately 18,000 sites/cell. The molecular weight was estimated to be in the range of 60-100 kDa and the amino acid sequence of hIL-18R revealed 100% identity to the IL-1RrP (Torigoe *et al.*, 1997). IL-1RrP has been considered as the functional constituent of IL-18R and therefore has been renamed as IL-18α. The precise mechanism, by which IL-18 acts, involves the binding of IL-18 to IL-18α, which in turn binds to IL-18β. This complex then recruits IL-18R-activating kinase (IRAK) and MyD88, an intracellular adapter molecule, resulting in the translocation of the NFκB transcription factor to the nucleus to undergo gene transcription (see Dinarello, 1999).

1.1.5 Synthesis of IL-1β: IL-1β converting enzyme (ICE)

Interleukin-1β converting enzyme (ICE) was initially isolated from monocytic cells in 1988 (Black *et al.*, 1988; Kostura *et al.*, 1989; Sleath *et al.*, 1990). It was identified as a cysteine protease which cleaves the inactive precursor for IL-1β (31 KDa), also known as pro-IL-1β, to induce a biologically active form of IL-1β (17 kDa). ICE has been cloned, purified and the specificity of its biological actions has been investigated. The purification of ICE was performed on a human monocytic cell line, THP.1, using high-performance liquid chromatography (HPLC). Various lines of evidence have shown that ICE is a true cysteine protease, whereby the enzyme is inhibited by diazomethylketone and iodoacetate, which are highly selective inhibitors of cysteine proteases (Thornberry et al., 1992). The same group established that ICE is a heterodimer that comprises two subunits, p20 and p10, which were encoded in a single 45 KDa precursor protein, described as p45. It is the p20 subunit that contains

the catalytic cysteine and kinetic analysis has shown that both subunits are necessary for catalytic activity (Thornberry *et al.*, 1992).

It has been reported that it is the dominant form of ICE, p45, which is located in the cytoplasm of THP.1 monocytic cells. Monocytic cells treated with lipopolysaccharide (LPS) did not express any pro-IL-1β cleavage activity, or any change or disruption in p45. This evidence suggests that in LPS-stimulated cells, active p20/p10 ICE is uncommon. In order for ICE to cleave pro-IL-1β, p45 must be degraded to p20/p10 (Ayala *et al.*, 1994). As p45 ICE has no substantial cleavage ability for pro-IL-1β, it was hypothesised that perhaps p20/p10 is synthesised within the cell and activity is induced by p45 (Ayala *et al.*, 1994).

Many groups have investigated a role for ICE in apoptosis, programmed cell death and neurodegeneration. Apoptosis is an essential biological process that allows cells to die in a controlled manner (see Nicholson & Thornberry, 1997). Reactive oxygen species, commonly known as free radicals have been implicated in a variety of biological changes including apoptosis (see Halliwell & Gutteridge, 1989). It has been proposed that the redox status within cells can influence apoptotic effects, because mediators of apoptosis, such as ICE are redox sensitive (Hampton *et al.*, 1998), although the precise mechanism whereby ICE is activated by oxidative stress is unclear (see Halliwell & Gutteridge, 1998).

Several experimental tools have been employed to ascertain the role for ICE. A mutant ICE gene (C285G) has been designed, which acts as a dominant negative ICE inhibitor. This gene has been shown to inhibit apoptosis by inhibiting the formation of mature IL-1β from pro-IL-1β. When transgenic mice expressing this gene are subjected to ischemic insult, neuronal viability is not compromised (Frielander *et al.*, 1997). The use of ICE-like protease inhibitors, such as N-benzlyoxycarbonyl-Val-Ala-Asp-fluromethylketone (z-VAD.FMK), and acetyl-Try-Val-Ala-Asp-chloro-methylketone (YVAD.CMK), have helped to elucidate the role of ICE further (Hara *et al.*, 1997). In general, many reports suggest that neurodegenerative diseases may be counter-acted by using these caspase inhibitors (see Frielander & Yuan, 1998). For example, ischemia or excitotoxic damage induced by intrastriatal micro-injection of NMDA or AMPA, can be blocked by intracerebroventricular (i.c.v.) of ICE inhibitors (Hara *et al.*, 1997).

1.1.6 Other cytokines

Although the IL-1 family has been characterized as playing a pivotal role in inflammatory and immune responses, there are a host of other cytokines that are key modulators of inflammation, infection and immunity. These cytokines participate in modulating these responses through a complex network of interactions (Feghali &Wright, 1997). Cytokines have been grouped into separate families in accordance with their structural and functional similarities. This group of polypeptides include interleukins, interferons, cell stimulating factors and tumour necrosis factors. Cytokines are generally produced by immune cells such as macrophages, lymphocytes and also fibroblasts in the peripheral nervous system and by neuronal and glial cells in the brain (Rothwell & Relton, 1993). Many of these cytokines are involved in mediating humoral immune responses such as IL-4, IL-5, IL-6, IL-7 and IL-13, while other are responsible for mediating cellular responses, for example IL-1, IL-2, IL-3, IL-4, IL-7 and IL-12 (see Fegahli & Wright, 1997). Proinflammatory cytokines include IL-1, IL-6, IL-8, tumour necrosis factor-α (TNFα), interferon-α (IFNα) and interferon-γ (IFNγ: see Turrin and Plata-Salamán, 2000). The proinflammatory cytokine network existing in the brain is quite complex and it is generally accepted that cytokines can act either synergistically or antagonistically. For example, it has been shown that IL-1β injected intracerebroventricularly evokes enhanced mRNA levels of TNFα and IL-6 in the rat liver and spleen (Kitamura *et al.*, 1998). Cytokines such as IL-4, IL-10, IL-13, transforming growth factor-β (TGFβ) and the IL-1ra have been classified as anti-inflammatory cytokines. The primary function of these cytokines appear to downregulate the adverse effects of proinflammatory cytokines or inflammation (Turrin & Plata-Salamán, 2000). For example, IL-1ra has been shown to exert a protective effect in rat cerebral ischemia in which inhibition of IL-1ra enhances ischemic damage (Loddick *et al.*, 1997). Studies carried out on polymorphonuclear leukocytes (PMN), specialized effector cells involved in acute inflammatory responses have examined the integrated role of proinflammatory and anti-inflammatory cytokines. IL-10 has been shown to modulate TNFα, IL-1β and IL-8 cytokine gene expression and production in PMN (Cassatella *et al.*, 1993). Further studies carried out by this group, have reported that the stimulant lipopolysaccahride (LPS), induces IL-1ra production and this increase in IL-1ra is further augmented by IL-10. From this finding, it was hypothesized that IL-

10 exerts a protective effect or anti-inflammatory effect against Gram-negative infections in which IL-1ra was preferentially increased instead of IL-1β (Cassatella *et al.*, 1994).

The importance of cytokine production has been exemplified in ongoing research to elucidate the role they play in neurological, inflammatory and immunological disorders. Probably one of the most studied groups of cytokines to date are the proinflammatory cytokines. The production of IL-1α, IL-1β and IL-1ra has been extensively studied since an increase in gene expression and synthesis has been observed in various diseased states such as ischemic diseases, Alzheimer's disease, asthma, leukemias, solid tumours and viral, parasitic, fungal and bacterial infections (see Dinarello, 1996).

1.2 Bological activities of IL-1β

1.2.2 IL-1β and neurodegeneration

IL-1 has been described as a multifunctional proinflammatory cytokine that affects most cell types. Research to date has focused on the role IL-1 plays in host defence responses to systemic diseases. IL-1 can evoke multiple responses which are capable of inhibiting, provoking or instigating neuronal damage and death (see Rothwell, 1999). Various methods have been employed to elucidate the effects of IL-1β on neurodegeneration. IL-1 expression has been measured after the onset of clinical and experimental brain damage, exogenous IL-1 has been investigated in these circumstances, and many studies have used inhibitors such as anti-IL-1 antibodies, IL-1ra, and inhibitors of IL-1 synthesis such as ICE to confirm the effects of IL-1. Administration of recombinant IL-1 (rIL-1) into the brain of experimental animals or to cultured neuronal cells evokes responses typical of brain injury, such as glial activation and proliferation (Loddick *et al.*, 1998).

Most studies indicate that IL-1β is the key member of the IL-1 family that plays a pivotal role in neurodegeneration in the brain. This cytokine has been implicated in several forms of brain degeneration such as ischemia, traumatic and excitotoxic brain injury (Pearson *et al.*, 1999). The production of IL-1 in disease has been extensively studied. By means of *in situ* hybridization, antibody staining of tissue, analysis of circulating levels and examination of mRNA levels, reports have showed that gene expression and synthesis of IL-1α, IL-1β and IL-1ra are augmented

in disease states. Patients suffering from Alzheimer's disease, HIV-1, asthma, viral, bacterial, parasitic and fungal infections, leukemias, graft-versus-host disease, UV radiation and many others, all show increased levels of IL-1 (see Dinarello, 1996).

Two extensively researched neurodegenerative disorders are Alzheimer's disease (AD) and Parkinson's disease (PD). Despite extensive research carried out on these two disorders, the cause of neuronal cell death that is associated with both remains to be elucidated. Concentrations of IL-1β have been measured in the CSF and plasma of PD and AD patients. An increased concentration of IL-1β was detected in plasma and CSF from both types of patients; this has been interpreted as evidence of the involvement of IL-1β in the acute phase response, contributing to lysis of degenerating neuronal cells (Blum-Degen *et al.,* 1995). The effect of IL-1β on patients with multiple sclerosis (MS) has also been studied. IL-1β has been detected in the CSF of MS patients and it is thought that, at high concentrations, IL-1β can act synergistically with other proinflammatory cytokines such as IL-6 and TNFα to contribute to demyelination and death of oligodendrocytes (Zhao & Schwartz, 1998). Evidence also implicates IL-1β in the progression of senile plaques, the β-amyloid rich structures associated with AD. Furthermore, it is thought that excess production of IL-1β and TNFα, as a result of acute-phase responses, induces the pathophysiology of AD by causing over activation of the β-amyloid precursor protein (β-APP, see Malek-Ahmadi, 1998). It has also been reported that prolonged over-expression of these cytokines can induce nitric oxide (NO) production, leading to neuronal cell death. Collectively, these studies support the theory that β-amyloid and cytokines act synergistically to induce inflammatory responses, thus playing a pivotal role in the neuropathology of AD (Zhao & Schwartz, 1998).

One of the most important factors that have been attributed to neurodegeneration is the release of endogenous excitatory amino acids (EAA's) such as glutamate and aspartate. In neurodegeneration, these EAA's act primarily on N-methyl-D-aspartate (NMDA) receptors. When these EAA's are present in high concentrations, they are capable of evoking neurotoxic effects (Choi & Rothman, 1990; Bullock &Fujisawa, 1992). NMDA receptor-mediated cell death is frequently used as an experimental model for neuronal damage subsequent to traumatic and ischemic brain degeneration. Infusion of the NMDA receptor agonist, cis-2,4-methanoglutamate (MGlu), in the rat cortex and striatum results in IL-1β induction in

glial cells (Pearson *et al.*, 1999). Exocitotoxins such as kainate or specific NMDA receptor agonists are capable of instigating rapid expression of IL-1β mRNA and protein in the brain which is comparable to responses following ischemia and brain trauma (Pearson *et al.*, 1998).

The use of inhibitors has been frequently employed to verify that IL-1β is involved in ischemia and other neurodegenerative disorders. Central administration of IL-1ra inhibits damage brought about by focal cerebral ischemia (middle cerebral artery occlusion (MCAo)). For example, it has been reported that intracerebroventricular (i.c.v.) injection of IL-1ra (10μg) 30 min prior and 10 min following ischemia, significantly reduces neuronal cell death that would otherwise have occurred (Relton & Rothwell, 1992). IL-1ra has also been used to examine the role of IL-1β in cortical and striatal damage. Infusion of the excitotoxins, MGlu and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (s-AMPA) caused an induction of NMDA and AMPA receptor activation, resulting in striatal and cortical lesions. Administration of hrIL-1ra in this study impaired striatal damage, but had no effect on neuronal degeneration or cortical damage. This may be due to the fact that higher concentrations of IL-1ra may be necessary, or due to the site of action. However, this evidence clearly shows that endogenous IL-1β appears to mediate this type of neurodegeneration *in vivo*.

The mechanism by which IL-1β exerts its effects appears to be receptor-mediated (Lawrence *et al.*, 1998). However, many other factors appear to be significantly involved in IL-1β-induced neuronal degeneration. For example, IL-1β may possibly induce or activate factors that may account for the neurotoxic effects that occur (see Rothwell, 1999). IL-1β has been reported to increase intracellular calcium concentrations in cultured cortical neurones (Campbell *et al.*, 1998), induce reactive oxygen species formation (Murray *et al.*, 1999) lipid peroxidation (Murray & Lynch, 1997). The extent of damage that IL-1β can induce may depend on the site of action, concentration, extracellular and intracellular stimuli (Touzani *et al.*, 1999).

1.2.2 IL-1β and neuroprotection

Most of the evidence to date demonstrates how IL-1 is implicated in neuronal viability, neurodegeneration and acute-host response. However there is also evidence to show that IL-1 can confer neuroprotection under particular circumstances. It has

been previously reported that TNFα is neuroprotective in cultured cortical neurons. This neuroprotectivity is receptor-mediated. The receptor involved is the p55/TNF receptor Type I (TNFR1). The same study also showed that the plant alkaloid nicotine inhibits TNFα-induced neuroprotection (Carlson *et al.*, 1998). In parallel with this study, the possibility of a role for IL-1β in neuroprotection has also been investigated. IL-1α or IL-1β confered neuroprotection, in a concentration-dependant manner, against NMDA-induced excitotoxicity in cultured cortical neurones. This finding was confirmed by applying high concentrations of IL-1ra and anti-NGF antibodies to the cultures. IL-1ra was shown to block the IL-1-induced responses. This finding suggests that IL-1β neuroprotection is receptor mediated (Carlson *et al.*, 1999).

It has been hypothesised that IL-1 exerts neuroprotective and neurotrophic effects by activating the glial-derived neuronal growth factors such as nerve growth factor (NGF) and fibroblast growth factor (FGF, Lindholm *et al.*, 1987, Strijbos & Rothwell, 1995). It was shown that IL-1β protects cortical neurons in primary cultures against NMDA and non-NMDA receptor-mediated toxicity. The use of neutralizing antibodies to NGF impaired the neuroprotectivity conferred by IL-1β, thus confirming that NGF acts as a neuroprotective agent for IL-1β (Rothwell & Strijbos, 1995). Other reports have provided support for the hypothesis that cytokines, growth factors and excitatory can interact to determine neuronal viability. The neurotoxic effects of kainate, glutamate, MNDA and AMPA to cultured cortical neurons can be overcome by pretreating with IL-1β or NGF, or both (Strijbos & Rothwell, 1995).

1.2.3 IL-1β and reactive oxygen species formation (ROS)

Recent reports suggest that the formation of ROS maybe a consequence of increased levels of IL-1 and the concurrent increase in IL-1β production and ROS formation has been thought to impact greatly on synaptic plasticity, cell signalling and the ageing process. Studies undertaken by Murray and Lynch (1998a) suggested a role for IL-1β in age- and stress-induced impairments in long-term potentiation (LTP). It was hypothesized that the underlying cause was an increase in ROS formation, a repercussion of IL-1β production. Further studies reported parallel changes in ROS formation and IL-1β production. For example, ROS formation was

shown to cause an increase in IL-1β production while IL-1β had the ability to induce ROS formation (O'Donnell *et al.*, 2000); this suggests the existence of a positive feedback loop which is potentially very damaging to cells. Further confirmation of this sequence of events was shown in an age-related study in which an IL-1β-induced increase in lipid peroxidation and ROS formation were observed in parallel (Murray *et al.*, 1997). Furthermore, an increase in endogenous IL-1β concentration occurred in parallel with an increase in lipid peroxidation in cortical tissue obtained from aged rats. These age-related changes were reversed by antioxidant vitamins, again confirming the theory that IL-1β and ROS act together to antagonise the ageing process (Lynch, 1999).

1.3 Reactive Oxygen Species (ROS)

1.3.1 Properties of ROS

In order for cells to survive, molecular oxygen is a fundamental requirement. However, by-products of this process, termed reactive oxygen species (ROS) or pro-oxidants, can induce damage to the cellular environment (Halliwell, 1992). ROS are free radicals which are intrinsic to normal human metabolism but which, in excess, can induce tissue damage or oxidative stress. This phenomenon may be described as an imbalance between existing between pro-oxidants and antioxidants (Fürst, 1998). Radicals are species containing one or more unpaired electrons such as: nitric oxide ($NO^{\bullet}$), superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$), all of which are capable of independent existence (Halliwell, 1992). Low levels of ROS are imperative for intracellular signalling in many biological processes, including cell differentiation, proliferation or apoptotic cell death, immunological responses and defence against bacterial infection. However, high doses of ROS coupled with the inability of the cell to deal with an abundant amount of ROS results in oxidative stress, consequently leading to cell malfunction (Mates *et al.*, 1999).

1.3.2 The anti-oxidant defence system

While ROS formation threatens the ability of the cell to function, the cell has evolved a defence system to cope with excess ROS formation. This defence system has been termed "the anti-oxidant defence system". Antioxidants function to scavenge or 'mop up' intermediate free radicals, and do so by inhibiting radicals from

attacking fatty acids side chains or membrane proteins (Halliwell, 1992). The system consists of enzymatic and non-enzymatic elements that function solely to neutralize ROS prior to ROS-inducing cellular damage (Matés *et al.*, 1999). These antioxidants include: superoxide dismutases (SODs), gluthatione peroxidase (GPx), catalase (CAT), tocopherols (vitamin E) and ascorbic acid (vitamin C).

SODs function by catalysing $O_2^{\bullet-}$ to the least reactive species, hydrogen peroxide (H_2O_2), which in turn is metabolized by CAT or GPx reactions (Fridovich, 1995). It is thought that SODs are vital to the antioxidant defence system and a protective role for SOD has been characterized in dopamine-induced apoptosis in human neuronal cells (Gabbay *et al.*, 1996) and brain tumours (Pu *et al.*, 1996). Similarly, CAT functions to fractionate H_2O_2 into water and oxygen (Yu, 1994). There is a high amount of this enzyme in the liver and kidney, whereas low amounts have been detected in the brain and it is generally thought that GPx is largely responsible for the decomposition of H_2O_2 in the brain. These enzymes act in concert exerting a protective effect whereby the SOD dismutation reaction catalyses H_2O_2 and CAT and GPx act to remove the end-product of dismutation (Pu *et al.*, 1996). For the purposes of this study, only two antioxidant scavengers, vitamin E and vitamin C, will be discussed in more detail.

1.3.3 Vitamin E and Vitamin C

Vitamin E and vitamin C have been considered to be the second line of defence that is used to deal with excessive ROS formation. Vitamin E is the primary antioxidant that exists in the membranes, whereas vitamin C is one of the major water-soluble antioxidants. The interaction between the vitamins has been investigated in rat hepaptocytes and the findings suggest that vitamin C function independently of vitamin E, reacting with radicals prior to vitamin E. This finding suggests that vitamin C indirectly prevents vitamin E loss (Glascott & Farber, 1999).

Vitamin E or α-tocopherol, was initially discovered in 1922 by Evans & Bishop, as a nutritional factor that was necessary for the prevention of reabsorption of the foetus in the adult rat (see Vatassary, 1992). The importance of vitamin E is well recognized for the maintenance of the structural and functional integrity of the human nervous system. However, it was not until 1962 that Tappel showed that vitamin E was an endogenous antioxidant that functioned to protect tissue lipids from the

deleterious effects of free radicals (see Sies & Stahl, 1995). Earlier studies by Pappenheimer & Goettsch in 1931 determined that vitamin E deficient diets resulted in cerebrellar encephalomalacia (see Vatassary, 1992). Vitamin E is not highly localized in the brain, but brain tissue concentration is highly conserved. Brain regions obtained from rats fed on a vitamin E enriched diet retained more vitamin E than peripheral regions where the concentration of vitamin E prior to the diet was significantly depleted (Vatassey *et al.,* 1994). Furthermore, vitamin E deficiency has been reported to result in a number of pathological changes in muscle, reproductive, cardiovascular and nervous systems (Vatassery, 1992).

Vitamin C or L-ascorbic acid, is a water soluble molecule which was discovered in 1928 as a reducing agent in the liver by Szent-György, and was identified four years later as an ingredient of lemon juice (see Grüwald, 1993). Vitamin C has often been characterized as a remarkable antioxidant based on its ability to act as a reducing agent, whereby it provides electrons for oxidants or other electron acceptors (Rumsey & Levine, 1998). Vitamin C traps peroxyl radicals prior to lipid peroxidation, thus protecting biomembranes against oxdative stress (Sies & Stahl, 1995). It is has been established that the transport of vitamin C to the brain is through a facilitative glucose transporter at the blood brain barrier. Vitamin C enters the brain in an oxidised form, known as dehydroascorbic acid, and is then reduced and retained as vitamin C (Agus *et al.,* 1997).

1.3.4 Dietary manipulation

Oxidative stress appears to be a serious consequence, in the case of many diseases, whereby excessive generation of free radicals induced more cellular injury than the initial insult. Antioxidant therapy has been the focus of many studies in recent decades. One of the most commonly tried therapies is dietary supplementation. It has been shown that vitamin E, implanted into the necks of rats for two weeks, either attenuated or prevented alcohol-induced cerebral vascular damage (Altura & Gebrewold, 1996). It has been reported that there was a reduced incidence of Alzheimer's disease in elderly patients given a diet supplemented with vitamin E and vitamin C (Morris *et al.,* 1998). Furthermore, vitamin E supplementation ameliorates exercise-induced oxidative stress in rat heart muscle (Goldfarb et al., 1996). Dietary manipulation has been used to assess the relationship between oxidative stress and

age-related changes in antioxidant defences in the rat cortex. Thus it was shown that vitamin E and vitamin C concentrations are significantly depleted in aged rat cortical tissue (O' Donnell & Lynch, 1998) while long term dietary supplementation with vitamin E and vitamin C reverses the age-related deficit in these antioxidants in the rat cortex (O' Donnell & Lynch, 1998). Another form of antioxidant therapy is through the use of polyunsaturated fatty acids (PUFA). PUFAs docosahexanoic acid (DHA) and arachidonic acid (AA) are highly susceptible to oxidation (Kalmijn *et al.,* 1997, McGahon *et al.,* 1999) and are present in high concentrations in cell membrane phospholipids. Dietary supplementation with DHA (McGahon *et al.,* 1999) and AA (McGahon *et al.,* 1997) have been shown to reverse age-related impairments in synaptic function in the rat, for example, the age-related decrease in glutamate release and LTP. Additionally, supplementation with fish oil, which is high in n-3 type PUFAs has been shown to attenuate anti-inflammatory effects in both humans and experimental animals and delays the onset of autoimmune responses, cardiovascular disease and cancer (see Fernandes *et al.,* 1996).

1.3.5 ROS and Stress

Stress is a common experience of everyday life and can be described as a condition that interferes with physiological and psychological homeostasis. The ability of organisms to cope sufficiently with internal and external stresses is a fundamental prerequisite for survival. In mammals, the limbic-hypothalamo-pituitary-adrenal (LHPA) system is the primary neuro-endocrine component of the stress response. Activation of this system is central to stress responses coupled with activation of adrenal glucocorticoids, which are prime mediators in behavioural and cell physiology (Fuchs & Flüge, 1998).

It has been thought that stress-induced ischemic-damage activates the production of ROS, resulting in tissue damage. Furthermore, stress has also been implicated in metabolic changes that may lead to oxidative tissue damage in the brain, a consequence of increased ROS formation (Liu & Mori, 1999). Studies on long-term emotional stress have shown it to be associated with an increase in GPx activity, but not SOD activity (Nilova *et al.,* 1993). From these reports is could be concluded that oxidative stress and ROS formation play a pivotal role in stress-associated changes.

1.3.6 ROS and Ageing

Harman (1981) described the ageing process in the following manner: "ageing is the progressive accumulation of changes with time associated with, or responsible for, the ever-increasing susceptibility to disease and death which accompanies advancing age". One theory of ageing that has been generally accepted, is the idea that accumulative damage induced by ROS over a lifetime gives rise to the ageing process. This theory was initially presented by Harman (1956) as the "free radical theory of ageing". Various studies support this hypothesis and have shown a correlation between ROS, ageing and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and stroke (see Butterfield *et al.,* 1999).

One of the most extensively studied processes is that of lipid peroxidation. Various groups have shown that this occurrence is prominent in aged-related studies. In aged rats increases in lipid peroxidation and ROS formation were coupled with a decrease in AA (Murray & Lynch, 1998a). Further studies have demonstrated that lipid peroxidation of fatty acids in membrane lipids can greatly influence the fluidity of the membranes (Choe *et al.*, 1995). A number of groups have described an age-related decrease in membrane fluidity and membrane PUFA, particularly AA (Lynch & Voss, 1994).

Considering the extent of the age-related changes that exist and the role that ROS play in initiating age-related changes, it follows that the antioxidant defence system is somehow unable to progress in a concomitant manner with the ageing process. In an attempt to address this question, various studies have investigated the antioxidant defence system in the aged brain. One report indicated that there is an age-related increase in SOD activity, coupled with a decrease in GPx and CAT activity in hippocampal tissue, suggesting that age is accompanied with an impaired antioxidant defence system. It follows that if an increase in SOD activity is not accompanied by parallel increases in activation of CAT and GPx activity, then the ability to adequately deal with free radicals is impaired (O' Donnell *et al.,* 2000).

Most studies to date have suggested that the ageing process might be delayed through dietary manipulation, though the cocktail of antioxidants needed to overcome the effects of ROS formation remains to be determined. It appears that the major underlying cause of ageing is an increase in ROS formation, although the question as to whether ROS is a cause or a consequence must be addressed (Nohl, 1993).

However, a cleavage product of lipid peroxidation, known as lipofuscin, is the earliest known "footprint" to suggest that increased ROS formation occurs concomitantly with ageing. Nevertheless, the sequence of events surrounding oxidative stress and ageing still remains to be elucidated (Nohl, 1993).

1.4 The Hippocampus

1.4.1 The structure of the hippocampus

The hippocampus is a region of the archicortex which has been the focus of much attention due to its reported involvement in the process of learning and retrieval of memory. The hippocampus consists of four regions within two C-shaped interlocking cell body layers. These sections are 1) the dentate gyrus, 2) the hippocampus proper which consists of areas CA1, CA2 and CA3 (Lorente de Nó, 1934), the subicular complex, which consists of the parasubiculum, the presubiculum and the subiculum, and 4) the entorhinal cortex.

The hippocampus has three main excitatory pathways running from the entorhinal cortex to the CA1 region. This "trisynaptic pathway" is a distinguishing feature of the hippocampus (Anderson, 1987) and consists of the perforant pathway, the mossy fibre pathway and the Schaffer collaterals (fig 1.1). The perforant pathway arises from the cells of the entorhinal cortex and synapses on the granule cells in the hilus of the dentate gyrus. The axons of the granule cells form the mossy fibre pathway, which synapse on the pyramidal cells of the CA3 region of the hippocampus. Finally, the axons of pyramidal cells in the CA3 region i.e. the Schaffer collaterals , synapse on the pyramidal cells in the CA1, which in turn projects back to the subiculum, and from there back to the entorhinal cortex again.

1.4.2 The role of the hippocampus

Since the 1950s, the hippocampus has been recognized as playing a role in the process of learning and memory. Scotville and Milner in 1957 described the anterograde amnesia suffered by the patient H.M who had undergone bilateral hippocampal removal as a treatment for epilepsy. Several other studies have demonstrated the importance of the hippocampus in the memory process. It was shown that lesions of the hipocampus result in impaired learning and memory, in both humans (Squire *et al.*, 1984) and animals (Orr & Berger, 1985). Additionally,

through magnetic resonance imaging and positron emission tomography, the involvement of the hippocampus in memory was identified, whereby analysis of blood flow changes and oxygen use was monitored during learning tasks (Squire *et al.,* 1990, Squire *et al.,* 1992).

A putative model for learning and memory known as long-term potentiation (LTP) is a form of synaptic plasticity and has been described in the hippocampus (Bliss & Lømo, 1973; Bliss & Gardner-Medwin, 1973). This putative model for learning and memory has been used extensively to elucidate the underlying mechanisms and cellular events associated with learning and memory in the hippocampus.

1.5 Long-term potentiation (LTP)

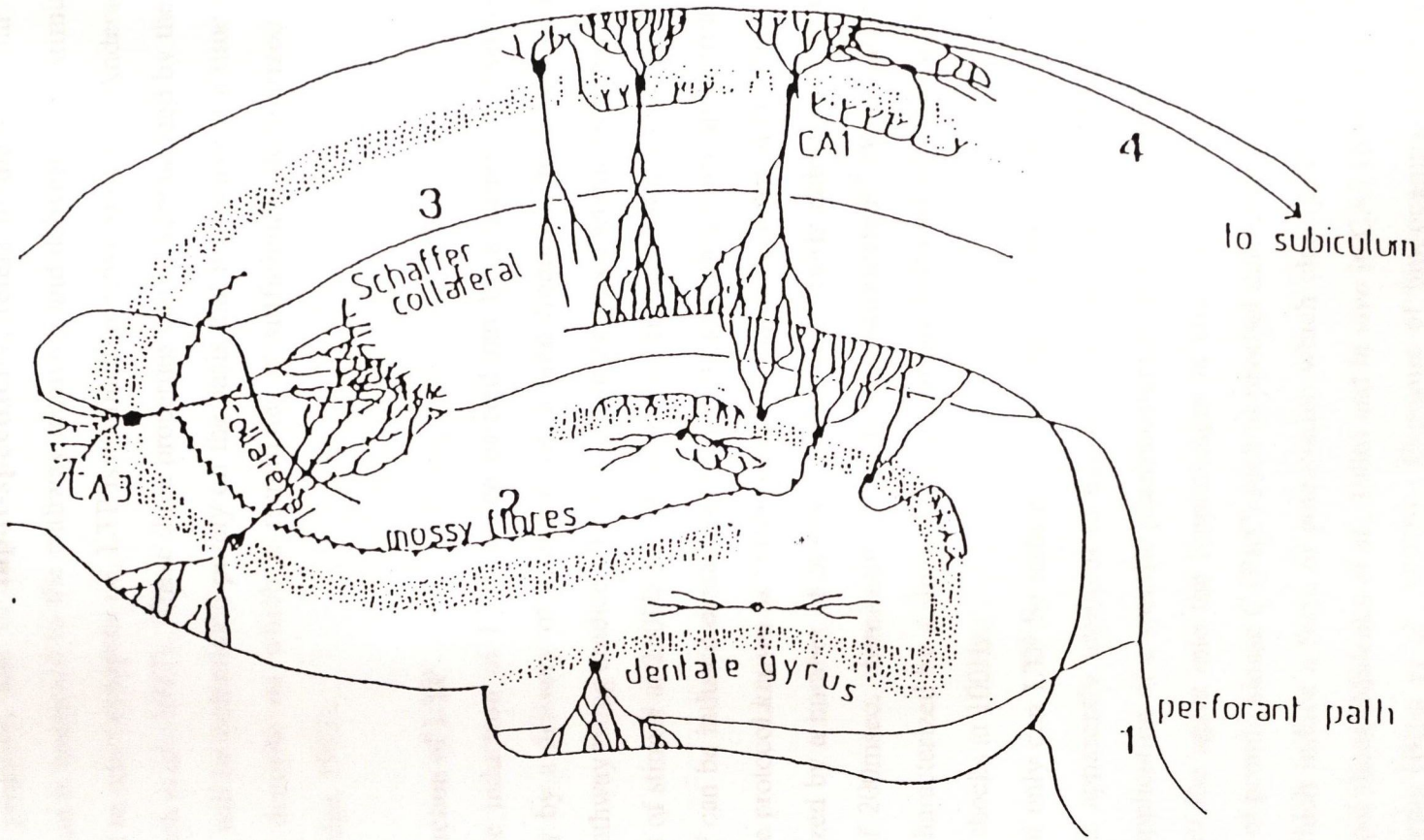
1.5.1 Properties of LTP

Long-term potentiation (LTP) in the hippocampus is probably one of the most salient examples of activity-dependent synaptic plasticity that has yet been identified in the mammalian brain. LTP is a phenomenon that was initially described by Bliss & Lømo (1973) in the perforant-path granule cell synapses of the anesthesized rabbit. They reported that brief trains of high frequency stimulation to the perforant path fibres resulted in a stable and long-lasting increase in the synaptic responses of the dendrites of the granule cells of the dentate gyrus. Since these findings, LTP has been described in the other main afferent pathways in the hippocampus and in other areas of the brain (Artola & Singer, 1993), namely cerebellar cortex, neocortex, striatum, amygdala, visual cortex and nucleus acumbens. As a result LTP can be broadly defined as the sustained increase in the amplitude of the synaptic response evoked in a single cell or populations of cells.

LTP is characterized by three essential properties namely, cooperativity, associativity and input specificity (see Bliss & Collingridge, 1993). **Cooperativity** refers to the existence of an intensity threshold for induction i.e a strong stimulus is necessary for the induction of LTP as a weak stimulus or a stimulus that is delivered for a relatively short period of time, will only activate a few afferent fibres and will not induce LTP (McNaughon *et al.,* 1978). This then suggests that the induction of LTP is dependent on the strength of the response of the postsynaptic neuron, which can be increased by cooperativity between active afferents converging on a

Fig 1.1 Anatomy of the hippocampus:

The following diagram illustrates a transverse section through the hippocampus of the rat. Inputs reach the hippocampus from the entorhinal cortex through the perforant path (1), which makes synapses with the dendrites of the granule cells of the dentate gyrus and also with apical dendrites of the CA3 pyramidal cells. The dentate granule cells project via the mossy fibers (2) to the CA3 pyramidal cells, which in turn project via the schaffer collaterals (3) to the CA1 area. CA1 contains pyramidal cells which send axons (4) to the subiculum.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postsynaptic site. The property of **associativity** relates to the idea that a weak stimulus can lead to potentiation if it is active concurrently with a strong stimulus to a separate but convergent input (Levy & Steward, 1979; McNaughton *et al.*, 1978). The final property, that of **input-specificity**, refers to the fact that synaptic transmission is restricted to the pathway receiving and delivering the stimulus does not extend to other synapses i.e. LTP is confined to active synapses (Anderson *et al.*, 1977; Lynch *et al.*, 1977). These three properties can be summarized by the fact that a synapse will be potentiated purely on the basis that it is active at a time when the region of dendrite on which it terminates is sufficiently depolarized (Bliss & Colloingridge, 1993).

1.5.2 Induction of LTP

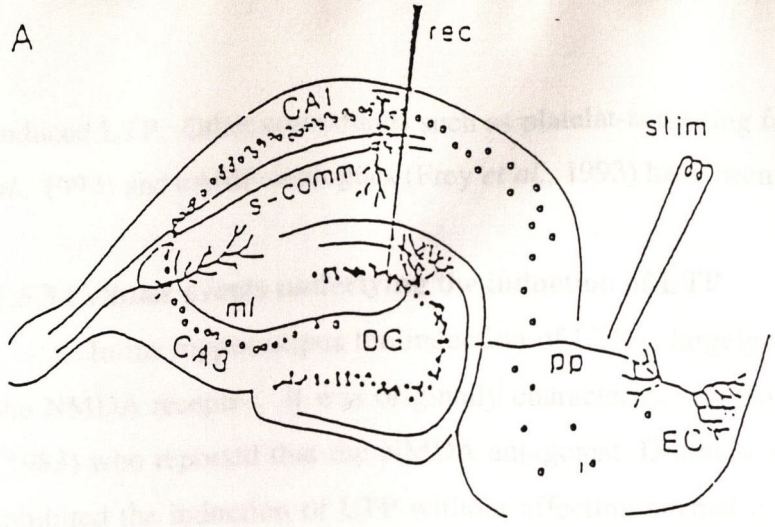
The induction of LTP can be carried out by a variety of methods, most commonly by a delivery of a tetanus i.e. a high frequency train of stimuli to the afferent pathway being studied. The present study uses a tetanic stimulation protocol of 3 trains of stimuli at 250Hz for 200msec with an inter-burst interval of 30sec (fig. 1.2). LTP can be induced electrically with more physiologically appropriate stimuli, namely the protocol known as "theta-burst stimulation" (Larson *et al.*, 1986) which is characterized by a number of bursts of 4 shocks at 100Hz delivered at an interburst interval of 200msec. Similarly, "primed-burst stimulation" (Rose & Dunwiddie, 1986) is characterized by a single priming stimulus followed at 200msec by a single burst of 4 shocks at 100Hz.

Not only can LTP be induced electrically, but also chemically in which the outcome is apparently identical to that of tetanically induced LTP. This method involves application of a specific pharmacological agent to the hippocampal slice or injection of the agent into the hippocampus *in vivo*. Among these compounds are activators of protein kinase C (PKC) such as phorbol diacetate and phorbol dibutyrate, both of which induce a form of potentiation which closely resembles LTP in CA1 hippocampal slices (Malenka *et al.*, 1986) and *in vivo* in CA3 (Bliss *et al.*, 1993) and dentate gyrus (Bliss *et al.*, 1997b). Exposure of hippocampal slices to elevated calcium or perfusion of the hippocampus with calcium was shown to induce a form of LTP (Turner *et al.*, 1982) in a manner remarkably similar to that of tetanically-

Fig 1.2 Example of LTP:

The following diagrams illustrate LTP in the dentate gyrus *in vivo*. A stimulating electrode was placed in the perforant path and a recording electrode was placed in the granule cell layer of the dentate gyrus (A). The graph plots the slope of the rising phase of the evoked response (population EPSP) recorded from the cell body region in response to test stimuli for 1 hour following the delivery of a tetanus (250Hz in 200msec), denoted by the arrow.

Taken from "A synaptic model of memory: long-term potentiation in the hippocampus". (1993) Bliss, T.V.P. and Collingridge, G.L. *Nature* **361** 31-39.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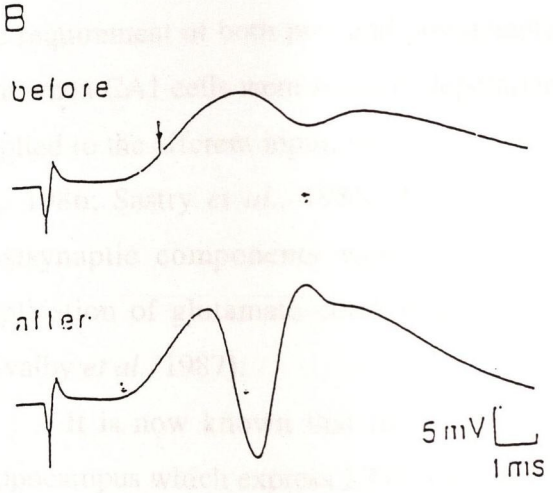
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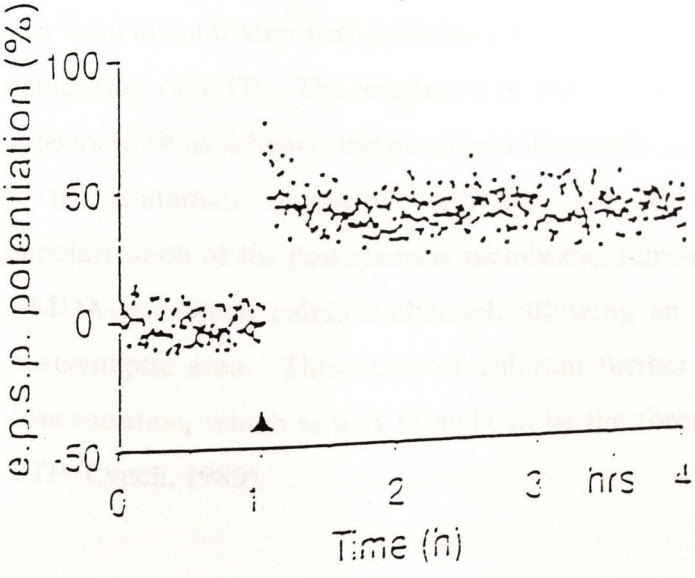
1 ms


e.p.s.p. potentiation (%)
100
50
0
-50
0
1
2
3
hrs
4
Time (h)

induced LTP. Other compounds such as platelat-activating factor (PAF; Wieraszko *et al.,* 1993) and cAMP analogues (Frey *et al.,* 1993) have been shown to induce LTP.

1.5.3 Cellular events underlying the induction of LTP

In the hippocampus the induction of LTP is largely attributed to activation of the NMDA receptor. It was originally characterized by Collingridge and colleagues (1983) who reported that the NMDA antagonist, D-amino phosphonovalerate (AP5), inhibited the induction of LTP without affecting normal transmission. This finding led to the belief that the induction of LTP was postsynaptically controlled. Lynch and colleagues (1983) discovered that injection of a calcium chelator, EGTA, into CA1 cells, inhibited LTP. Simultaneous reports described an experiment which suggested the requirement of both pre- and postsynaptic elements in the induction of LTP such that when CA1 cells were strongly depolarized at a time when single test shocks were applied to the afferent input, the response to the test shocks was potentiated (Kelso *et al.,* 1986; Sastry *et al.,* 1986; Wigstrom *et al.,* 1986). The role of both pre-and postsynaptic components was further suggested by the finding that dendritic application of glutamate combined with single afferent inputs also induced LTP (Hvalby *et al.,* 1987).

It is now known that the induction of LTP in the various subfields of the hippocampus which express LTP, with the exception of mossy fibre-CA3 synapses, is NMDA dependent. The significance of NMDA-receptor activation in induction of LTP is that calcium influx into the postsynaptic cell occurs through the NMDA-associated cation channel (McDermott *et al.,* 1986). The entry of calcium into the cell is a fundamental step that initiates a cascade of biochemical events leading to the expression of LTP. The sequence of events that underlies the induction of LTP appears to be as follows: tetanic stimulation induces release of glutamate which binds to the glutamate receptors, including the NMDA receptors. This leads to depolarization of the postsynaptic membrane, removing the magnesium block on the NMDA-associated calcium channel, allowing an influx of calcium ions into the postsynaptic area. This entry of calcium further augments intracellular calcium concentration, which now is thought to be the foremost trigger for the induction of LTP (Lynch, 1989).

1.5.4 The maintenance of LTP and associated cellular events

Although the induction of LTP has been well defined, the maintenance phase of LTP is less well understood and the locus of expression is still debatable. The experimental data emerging lead to the view that LTP involves profound changes in second messenger signalling both pre- and post-synaptically. Several reports from various groups have described a variety of biochemical and neurochemical changes that are thought to contribute to the maintenance of LTP, among which are neurotransmitter release, receptor binding, uptake and storage of calcium, protein phosphorylation, and phospholipid turnover.

One of the most widely studied biochemical changes that transpire as a consequence of LTP is an increase in the release of glutamate. Numerous reports have described that release of glutamate is tightly coupled with the induction of LTP, and therefore, at least partly contributes to the maintenance of LTP. The role of glutamate release will be discussed in more detail in the next section (1.5.5).

Several pieces of evidences exists which suggest the involvement of phospholipase C (PLC) in the expression of LTP. Firstly, McGahon and Lynch (1996) showed that the expression of LTP is associated with an increase in membrane inositol phospholipid metabolism, a process which involves PLC activation. It was also shown that arachidonic acid, a proposed retrograde messenger, increases the activation of PLC, as does the induction of LTP, in the dentate gyrus (McGahon & Lynch, 1998). A role for calcium/calmodulin kinase II (CaM kinase II), which is activated by binding of calcium/calmodulin in the expression of LTP, has been described by various groups. Postsynaptic microinjection of CaM Kinase II inhibitors have been shown to block the induction of LTP (Ito *et al.,* 1991). Furthermore, an increase in the activity of CaM kinase II has been observed in the induction of LTP (Malinow *et al.,* 1989).

The requirement of tyrosine kinase for the induction of LTP has been explored and the findings show that tyrosine kinase inhibitors block the induction of LTP (O' Dell *et al.,* 1991; Mullany & Lynch, 1997a). These inhibitors blocked the induction of LTP in CA1 in vitro, but had no effect on established LTP (O' Dell *et al.,* 1991), suggesting that tyrosine kinase plays a role in coupling the induction of LTP to the maintenance of LTP. A presynaptic role for tyrosine kinase in LTP has been described. The synaptic vesicle protein synaptophysin is the major presynaptic

substrate for tyrosine kinase and it has been shown that LTP is accompanied by increased phosphorylation of synaptophysin (Mullany & Lynch, 1998). Studies have also suggested that tyrosine kinase phosphorylation plays a role in the expression of LTP, whereby NGF interacts with the tyrosine kinase receptor, *trkA*, to modulate glutamate release and calcium influx (Maguire *et al.,* 1999).

Since the trigger for LTP is most likely to be the entry of calcium into the postsynaptic areas through the NMDA-associated calcium channels, and the maintenance of LTP is dependent on transmitter release, the existence of a retrograde messenger has been proposed. There are three prerequisites for a retrograde messenger: firstly, that it is released from the postsynaptic cell in response to tetanic stimulation or following application of glutamate; secondly, that it appears in perfusate following induction of LTP and finally that it acts on the presynaptic terminals to evokes an increase in neurotransmitter release (Lynch, *et al.,* 1991). One promising candidate for the role as a retrograde messenger is the polyunsaturated fatty acid arachidonic acid (AA) as it satisfies all the criteria necessary for a retrograde messenger. Firstly, NMDA-receptor activation stimulated the release of AA from hippocampal slices (Clements *et al.,* 1991). A sustained increase in the concentration of AA has been shown in perfusates of the dentate gyrus following induction of LTP (Clements *et al.,* 1991). Finally, it has been observed that AA and ACPD act synergistically to enhance glutamate release in rat dentate gyrus synaptosomes (McGahon & Lynch, 1994). Other proposed candidates for retrograde messengers are platelet-activating factor (PAF), a membrane-derived second messenger, neutrophins such as nerve growth factor (NGF), nitric oxide (NO) and carbon monoxide (CO). However the roles that these proposed retrograde messengers may play in the expression of LTP remain to be elucidated.

A role for mitogen-activated protein kinases (MAPKs) in the expression of LTP has been described recently. It has been observed that the p42 ERK isoform, but not the p44 isoform is activated in the area CA1 following direct stimulation of two components necessary for the induction of LTP, namely, PKC and NMDA (English & Sweatt, 1996). Furthermore, this study also demonstrated that ERK is activated in tetanically-induced LTP. The same group showed that the expression of LTP was impaired by the ERK inhibitor, PD 098095. Thus it was shown that inhibition of ERK inhibited the induction of LTP in area CA1, but not previously induced LTP

(English & Sweatt, 1997). Consistent with these reports is the observation that ERK activation was increased by AA and ACPD *in vitro,* and following the induction of LTP in perforant-path granule cell synapses. The same study also observed that the concomitant increase in the activation of ERK, release of glutamate and expression of LTP was inhibited by the ERK inhibitor, PD098095 (McGahon *et al.,* 1999).

1.5.5 Glutamate as a neurotransmitter

Glutamate is a major excitatory neurotransmitter in the central nervous system and is present in high concentrations throughout the brain (Fonnum, 1984). It is present within neurons and appears to be compartmentalized into two pools, the metabolic pool and the vesicular pool. Most available evidence, especially the finding that release is calcium-dependent (Sandoval *et al.,* 1978) suggests that the release of glutamate is of vesicular origin. Glutamate fulfils the criteria necessary for a neurotransmitter: firstly, glutamate is synthesized and stored in the nerve terminal, secondly, uptake occurs either from glucose via the Krebs cycle or from metabolized glutamine which is synthesized and transported to the neuron from glial cells (Reubi, 1980). Thirdly, there are mechanisms in existence, which allow the re-uptake of glutamate from the synaptic cleft, thereby terminating its action as a neurotransmitter (Nicholls & Atwell, 1990).

Convincing evidence exists that glutamate is a transmitter in the hippocampus. This has arisen largely from electrophysiological studies in which excitatory responses were recorded in hippocampal neurons following application of glutamate (Dudar, 1974). Further studies observed that glutamate release was stimulus- and calcium dependent (Nadler *et al.,* 1976) while lesoning of the hippocampus was associated with a decrease in the release of glutamate (Nadler & Smith, 1981).

1.5.6 Glutamate release and LTP

The synaptosomal preparation (Whittaker & Gray, 1962) is a commonly employed tool to investigate neurotransmitter release and presynaptic function, and hence was used throughout this study. Preparation of synaptosomes involves homogenization of the brain tissue in an iso-osmotic sucrose solution followed by centrifugation. The neck of the axon at the point where it enters the terminal is fragile and readily pinches off resulting in a resealed intact isolated nerve terminal or

synaptosome; the preparation is largely depleted of postsynaptic elements. Following its preparation, the synaptosome retains all the machinery necessary for the uptake, storage and release of glutamate (Nicholls, 1993).

One of the arguments that the maintenance of LTP involves the presynaptic terminal arises from the observation that an increase in glutamate release is associated with expression of LTP in dentate gyrus, which is the hippocampal subfield of interest here. The LTP-associated increase in glutamate release was originally observed by Skrede and Malthe-Sorenssen (1981), who observed that unstimulated release of radiolabelled D-aspartate, a marker of glutamate, was increased subsequent to tetanic stimulation. Later, using a combination of push-pull cannulation, electrophysiological recordings (Errington *et al.,* 1983) and radiolabelling, it was observed that there was an augmentation of newly synthesized glutamate (Dolphin *et al.,* 1982) which was associated with the expression of LTP. This finding was confirmed by studies that analysed endogenous glutamate release (Bliss *et al.,* 1986a; Errington *et al.,* 1987; Lynch *et al.,* 1989). Other studies used an *ex vivo* model, which involved analyses of glutamate release in synaptosomes prepared from dentate gyrus in which LTP had been induced *in vivo.* The results revealed a correlation between the expression of LTP and an increase in radiolabelled (McGahon & Lynch, 1996a; McGahon *et al.,* 1997) or endogenous (Canevari *et al.,* 1994) glutamate. The observation that the inhibition of LTP by electrophysiological (Bliss *et al.,* 1986) or chemical (Errington *et al.,* 1987; Lynch *et al.,* 1989b) means, inhibits this increase in glutamate, is further evidence of a tight coupling between glutamate release and LTP. It could be hypothesised that the maintenance of LTP relies at least to some extent on this presynaptic increase in glutamate release, which has been one of the most consistent observations associated with the expression of LTP.

1.5.7 Ageing and LTP

It has been documented on many occasions that aged rats have a reduced ability to sustain LTP (Landfield *et al.,* 1978; Barnes, 1979; Barnes & McNaughton, 1985; McGahon & Lynch, 1997, Murray & Lynch, 1998a,b). Earlier studies identified that there were behavioural deficits associated with age (Barnes, 1979) and data suggest that the expression of LTP in CA1 area in aged rats is associated with spatial learning capabilities (Diana *et al.,* 1994; Diana *et al.,* 1995). Although there is

a general acceptance that the ability of aged rats to sustain LTP is considerably impaired, the question whether there is an age-related change in induction of LTP remains unclear, with evidence for a deficit reported by some (Lynch &Voss, 1994; McGahon *et al.,* 1997; Murray & Lynch, 1998b) and no change reported by others (Barnes, 1979; Dupree *et al.,* 1993). Among the age-related changes that may contribute to impaired LTP is an increase in lipid peroxidation in the hippocampus which is coupled with a decrease in AA (Lynch *et al.,* 1994) and glutamate release (Mullany *et al.,* 1997) in the rat dentate gyrus (McGahon *et al.,* 1997).

One of the explanations that has been offered to account for these age-related impairments is the associated decrease in membrane AA concentration in the hippocampus (McGahon *et al.,* 1997) and dentate gyrus (Lynch & Voss, 1994; Murray & Lynch, 1998a, b) which may directly affect membrane fluidity and subsequently membrane function. Furthermore, it has been demonstrated that a decrease in phospholipase enzyme activity, which is necessary for the release of AA from membrane phospholipids, is reduced with age. This directly impacts on AA concentration and its ability to interact with ACPD, thus contributing to the age-related impairment in LTP (Lynch & Voss, 1994). To confirm that AA plays a pivotal role in sustained LTP, it was demonstrated that restoring membrane AA reverses the age-related impairment in LTP (McGahon *et al.,* 1997). Rats fed on a diet enriched with AA and its precursor γ-linolenic acid for 8 weeks, were assessed for their ability to sustain LTP. Analysis revealed that the dietary supplementation reversed the age-related impairment in LTP which was coupled with an increase in membrane AA concentration and an increase in glutamate release (McGahon *et al.,* 1997).

One of the proposed triggers for a decrease in membrane AA concentration is ROS as neuronal tissue is highly susceptible to oxidative stress (Halliwell, 1992). This hypothesis has been tested in relation to age-related impaired LTP. Aged rats fed on a diet-enriched with antioxidants, vitamin C and vitamin E, sustained LTP in rat dentate gyrus, unlike rats that were fed on a control diet (Murray & Lynch, 1998b). It was also determined that the vitamin-enriched diet reversed the age-related increase in lipid peroxidation. From these reports, it appears that the integrity of the membrane and the biophysical properties that are involved, such as ion channels and pumps, protein synthesis, activities of kinases and lipids, are essential for the

successful expression of LTP. Any compromise in these membrane properties appears to impact on changes associated with LTP.

1.5.8 IL-1β and LTP

Over the past decade it has become clear that IL-1β plays a critical role in neurodegenerative disorders (Dinarello, 1996) and has been shown to inhibit the expression of LTP in mossy fiber-CA3 pathway in the mouse hippocampus *in vitro* (Katsuki *et al.,* 1990). Later, a similar finding indicated that IL-1β inhibited both synaptic strength and LTP in Schaffer-collateral–CA1 hippocampal slices (Bellinger *et al.,* 1993). More recent studies illustrated the inhibitory effect of IL-1β on LTP in rat dentate gyrus *in vitro* (Cunningham *et al.,* 1996) and *in vivo* (Murray & Lynch, 1998a). The IL-1β-induced inhibition on LTP in rat dentate gyrus was reversed by co-applying IL-1β with its receptor antagonist, IL-1ra (Cunningham *et al.,* 1996).

In parallel with the inhibitory effect of IL-1β on LTP, the data from Cunningham *et al.* (1996) indicated that IL-1β inhibits the LTP-induced increase in calcium influx into hippocampal slices. These studies are supported by the finding that IL-1β reduces calcium channel currents in dissociated adult guinea-pig hippocampal slices (Plata-Salamán & Ffrench-Mullen, 1994) and calcium-channel currents in cultured cortical neurons (MacManus *et al.,* 2000). These studies are consistent with the idea that IL-1β may inhibit LTP via calcium mediated mechanisms. Schneider and colleagues (1998) reported that the induction of LTP in hippocampal slices and in the hippocampus of freely moving rats is coupled with an increase in the gene expression of IL-1β. Application of IL-1ra resulted in impaired LTP; these two results suggest that IL-1β play a role in the maintenance of LTP that appears to conflict with previous data. However, these differences could be attributed to IL-1β concentration. It is possible that application or injection of IL-1β may mimic increases in the concentration of IL-1β, in the brain following infection or inflammation. However, the study carried out by Schneider (1998) analysed the concentration of IL-1β in the "healthy brain" where basal levels of IL-1β might be expected.

Further studies have attempted to elucidate the underlying cause of the IL-1β-induced inhibition in LTP. One possible contributing factor is that it leads to an increase in the formation of ROS. One recent study has shown that, associated with

the age-related impairment in LTP, is an increase in IL-1β concentration (Murray & Lynch, 1998a). To determine whether ROS formation was causally related to an increase in IL-1β, the same group fed aged rats on a long-term anti-oxidant diet enriched with vitamin E and vitamin C. Their ability to sustain LTP was assessed, and the results revealed that rats fed on the diet were capable of sustaining LTP, unlike aged rats fed on the control diet (Murray & Lynch, 1998b). This data provided strong evidence that an increase in ROS formation was a possible causative link between the increase in IL-1β concentration and the age-related impairment in LTP.

1.6 The mitogen-activated protein kinase (MAPK) superfamily

1.6.1 ERK, JNK and p38

The mitogen-activated protein kinase (MAPK) superfamily of enzymes plays a critical role in signal transduction. This superfamily is comprised of four distinct subgroups: extra-cellular signal-regulated kinases (ERK's), c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK1), ERK5/big MAP kinase-1 (BMK1) and the p38 group of kinases (Ono *et al.,* 2000). These proteins are involved in integrating signals from upstream activators and transferring them downstream to other targets and effector proteins (see Egan & Weinberg, 1993). Each of the kinases responds differently to various extracellular and intracellular stimuli. The ERK subfamily of kinases are activated by a number of growth factors; including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF) and serum (Cano & Mahadevan, 1995; Ferrell & Bhott, 1997). The BMK1 kinase acts by regulating early gene expression in response to serum (Kato *et al.,* 1997). The JNK and p38 subfamilies are activated by a different type of stimuli such as: toxic chemicals, irradiation, proinflammatory cytokines, osmotic shock and ultra violet light (Raingeaud *et al.,* 1995; Tibbles & Woodgett, 1999).

1.6.2 Nomenclature and Isoforms

The ERK protein was originally termed Microtubule-Associated Protein Kinase but was later termed Mitogen-Activated Protein Kinase (MAPK; see Jarvis & Schaefer, 1997). The terms ERK and MAPK, which are often used interchangeably, describe two isoforms, p44 ERK1 and p42 ERK2 (Cano & Mahadevan, 1995; Neary, 1997). The JNK protein kinases are encoded by three genes: *jnk1* and *jnk2*, which are

expressed ubiquitously and *jnk3*, which is expressed in the brain, heart and testis (see Ip & Davis, 1998). The major isoforms of JNK are p46 JNK1, p54 JNK2 and p49 JNK3 (Kyriakis *et al.,* 1994). The p38 kinase was formerly known as Cytokine Suppressive Anti-inflammatory Drug Binding Protein or CSBP. Other groups have also designated this protein SAPK2a, SAPK2, Mxi2 and RK. Five mammalian isoforms of the p38 subfamily have been identified as p38/p38α, p38β2, p38γ, p38δ/SAPK4 and SAPK3 (Han *et al.,* 1994; Enslen *et al.,* 1998; Li *et al.,* 1996; Cuenda *et al.,* 1997; Goedert *et al.,* 1997). The nomenclature for these kinases is extremely varied, so for clarity, these protein kinases will be referred to as JNK and p38 throughout this thesis.

1.6.3 Signalling pathways-JNK and p38

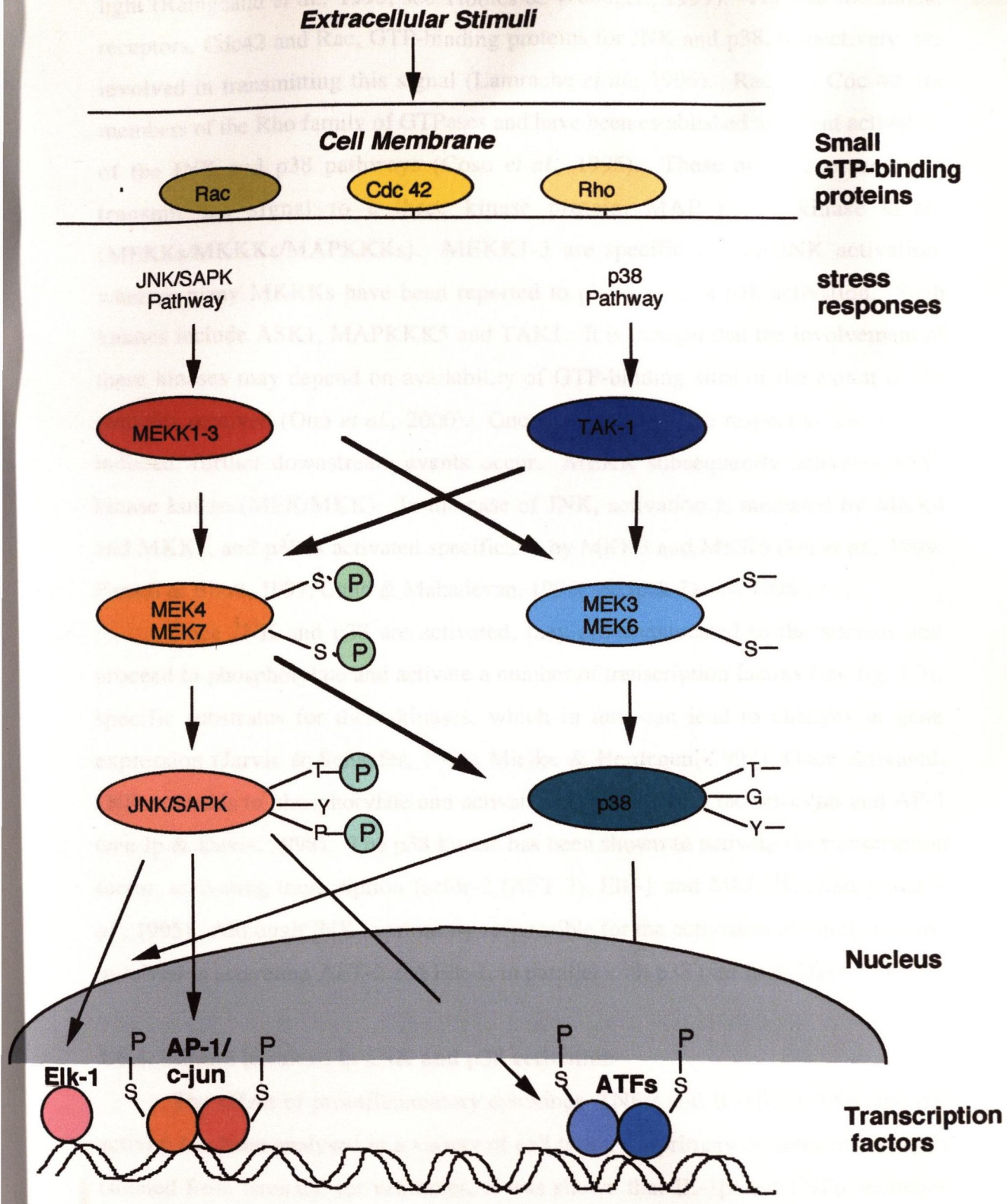
For the purposes of this study, it is JNK and p38 that are of interest and therefore these will be discussed in more detail. Among the major tools used to elucidate the role the MAPK superfamily plays in signal transduction are proinflammatory cytokines such as TNFα and IL-1β. These cytokines have been established as potent inducers of the JNK and p38 pathways, but do not activate ERK (Raingeaud *et al.,* 1995; Uciechowski *et al.,* 1996). JNK and p38 are implicated in diverse biological processes such as apoptosis (Nagata & Todokoro, 1999), stress responses (Chen *et al.*, 1996) and induction of stress-induced immediate early genes (Lim *et al.,* 1998). A range a cellular responses are also induced, such as repair, differentiation, development, growth, transformation and inflammatory responses (see Tibbles & Woodgett, 1999). The methods by which the JNK and p38 pathways are activated have been studied extensively: kinetic and mechanistic studies have established that MAPKs are dually phosphorylated on tyrosine and threonine residues within the kinases activation loop. This phosphorylation event is crucial in order for enzyme activity to occur (Payne *et al.,* 1991; Zhang *et al.,* 1995) and this characteristic is often used as a prerequisite to determine members of the MAPK superfamily (Raingeaud *et al.*, 1995).

The signalling events that occur within the JNK and p38 pathways can be described as follows: JNK and p38 are initiated upon receiving a stressful extracellular stimuli, such as those previously mentioned, for example, toxic chemicals, irradiation, proinflammatory cytokines, osmotic shock and ultra-violet

Fig 1.3 The stress-activated protein kinase signalling pathways:

JNK and p38 are members of the MAPK superfamily and play a fundamental role in cell signal transduction. The pathways are activated upon receiving appropriate extracellular or intracellular stimuli where the kinases become phosphorylated and thereby activate the next member of the cascade and activation of these kinases often results in 'interwoven ' pathways. In each case, activation of these cascades may be restricted to the cytosol or it can extend to the nucleus, where activation of transcription factors can lead to changes in gene expression.

Adapted from "Detection of JNK, p38 and MAPK enzyme activation using anti-dual-phosphopeptide antibodies: Coordinated signalling of the extracellular signal-regulated protein kinase (ERK) superfamily". (1996) Jarvis, B.W. and Schaefer, E.M. *Promega notes* 2-6.


Extracellular Stimuli
Cell Membrane
Rac
Cdc 42
Rho
Small GTP-binding proteins
JNK/SAPK Pathway
p38 Pathway
stress responses
MEKK1-3
TAK-1
MEK4 MEK7
MEK3 MEK6
JNK/SAPK
p38
Nucleus
Elk-1
AP-1/ c-jun
ATFs
Transcription factors

light (Raingeaud *et al.,* 1995; see Tibbles & Woodgett, 1999). The cell membrane receptors, Cdc42 and Rac, GTP-binding proteins for JNK and p38, respectively, are involved in transmitting this signal (Lamrache *et al.,* 1996). Rac and Cdc 42 are members of the Rho family of GTPases and have been established as potent activators of the JNK and p38 pathways (Coso *et al.,* 1995). These membrane receptors transmit the signal to a three kinase protein, MAP kinase kinase kinase (MEKKs/MKKKs/MAPKKKs). MEKK1-3 are specific for the JNK activation, whereas many MKKKs have been reported to play a role in p38 activation. Such kinases include ASK1, MAPKKK5 and TAK1. It is thought that the involvement of these kinases may depend on availability of GTP-binding sites or the extent of the stimulus received (Ono *et al.,* 2000). Once activation of the respective kinases are induced, further downstream events occur. MEKK subsequently activates MAP kinase kinase (MEK/MKK). In the case of JNK, activation is mediated by MKK4 and MKK7, and p38 is activated specifically by MKK3 and MKK6 (Hu *et al.,* 1999; Ferrell & Bhott, 1997; Cano & Mahadevan, 1995; see Ip & Davis, 1998).

Once JNK and p38 are activated, they are translocated to the nucleus and proceed to phosphorylate and activate a number of transcription factors (see fig. 1.3), specific substrates for these kinases, which in turn can lead to changes in gene expression (Jarvis & Schaefer, 1996; Mielke & Herdegen, 1999). Once activated, JNK proceeds to phosphorylate and activate the transcription factors c-jun and AP-1 (see Ip & Davis, 1998). The p38 kinase has been shown to activate the transcription factor, activating transcription factor-2 (AFT-2), Elk-1 and MEF-2C (Raingeaud *et al.,* 1995). Although JNK is primarily responsible for the activation of c-jun, it is also involved in activating AFT-2 and Elk-1, in parallel with p38 (see Ip & Davis, 1998).

1.6.4. Stimuli involved in JNK and p38 activation

The effect of proinflammatory cytokines, TNFα and IL-1β, on JNK and p38 activity has been analysed in a variety of cell types. In primary cultures of myocytes isolated from neonatal rat ventricles, it was shown that IL-1β and TNFα increased JNK and p38 activation and their respective transcription factors, c-jun and AFT-2. However IL-1β induced a more potent response than TNFα (Clerk *et al,* 1999). Similar activation has also been shown in human neuroma fibroblasts (Lu *et al.*, 1997) whereby cultured fibroblasts stimulated with TNF α and IL-1β, activate ERK and

SAPKs. This evidence suggests that theses two cytokines activate parallel signalling pathways in human neuroma fibroblasts, leading to fibroblast proliferation and formation of neuromas (Lu *et al.,* 1997). In order to distinguish between the two major signalling pathways, IL-1β-treated human glomerular mesangial cells (HMC) were assessed for JNK and ERK activity. The results showed that IL-1β stimulated activity of two JNK isoforms, JNK1 and JNK2 in these cell types, but not ERK. Many reports suggest that the activity of these kinases depends on the cell type, species, and the type of stimulation (Uciechowski *et al.,* 1996).

One of the factors that are associated with activation of SAPKs is osmotic shock (Cuenda *et al.,* 1997). Recent results show that exposure of renal epithelial A6 cells to a high potassium iso-osmotic solution causes activation of SAPKs. However, it appears that it is the changes in membrane tension, induced by cell swelling, that induces SAPK activation, and not osmolarity (Niisato *et al.,* 1999). Other forms of stresses, for example ultraviolet light C and gamma (γ) radiation, cause JNK activation. *In vitro* studies have shown that JNK1 is activated in Jurkat cells when they are exposed to a lethal dose of γ radiation. In parallel with these findings it was also shown that apoptosis was induced with γ radiation, suggesting a correlation between JNK activation and apoptosis under these circumstances (Chen *et al.,* 1996). Further studies revealed that only JNK, but not p38 or ERK, was activated by γ radiation and ultraviolet light C. Also, prolonged activation of JNK was correlated with apoptosis in Jurkat cells (Cheng *et al.*, 1996). Benzo(a)pyrene (BaP), a procarcinogen produced during the combustion of fossil fuels and cigarette smoke (see Philips, 1983), activated JNK and caspase-3 in Hepa 1c 1c 7 cells in a dose- and time- dependant manner. However, the relationship between caspases and JNKs still remains ambiguous (Rong *et al.,* 1998) with evidence which indicates that JNK can be both pro-apoptotic and anti-apoptotic. Thus, the JNK isoforms, JNK1, JNK2 and JNK3, may not mediate the same signals. It may be the case that both JNK and p38 signalling pathways are necessary for the activation of transcription factors implicated in apoptotic signalling (Ip & Davis, 1998). The activity of JNK, p38 and ERK, and their respective substrates have been assessed in rat cortex and hippocampus following systemic application of kainate. Kainate-induced excitotoxicity, increased JNK activation, phosphorylation of c-jun and AFT-2. However, p38 and ERK were

down regulated, therefore suggesting independent regulation of the p38 and JNK signalling pathways (Mielke *et al.*, 1999).

Much of the current evidence suggests that both kinases can act either independently of each other or concurrently. The regulation of the cascades appears to be due to many different downstream signals, and the literature suggests that cross-talk occurs between the two pathways, rather than individual pathways being activated in a straight forward stepwise manner (Ono *et al.*, 2000).

1.6.5 Related cell signalling events: IL-1β, IRAK, JNK, p38 and transcription factors

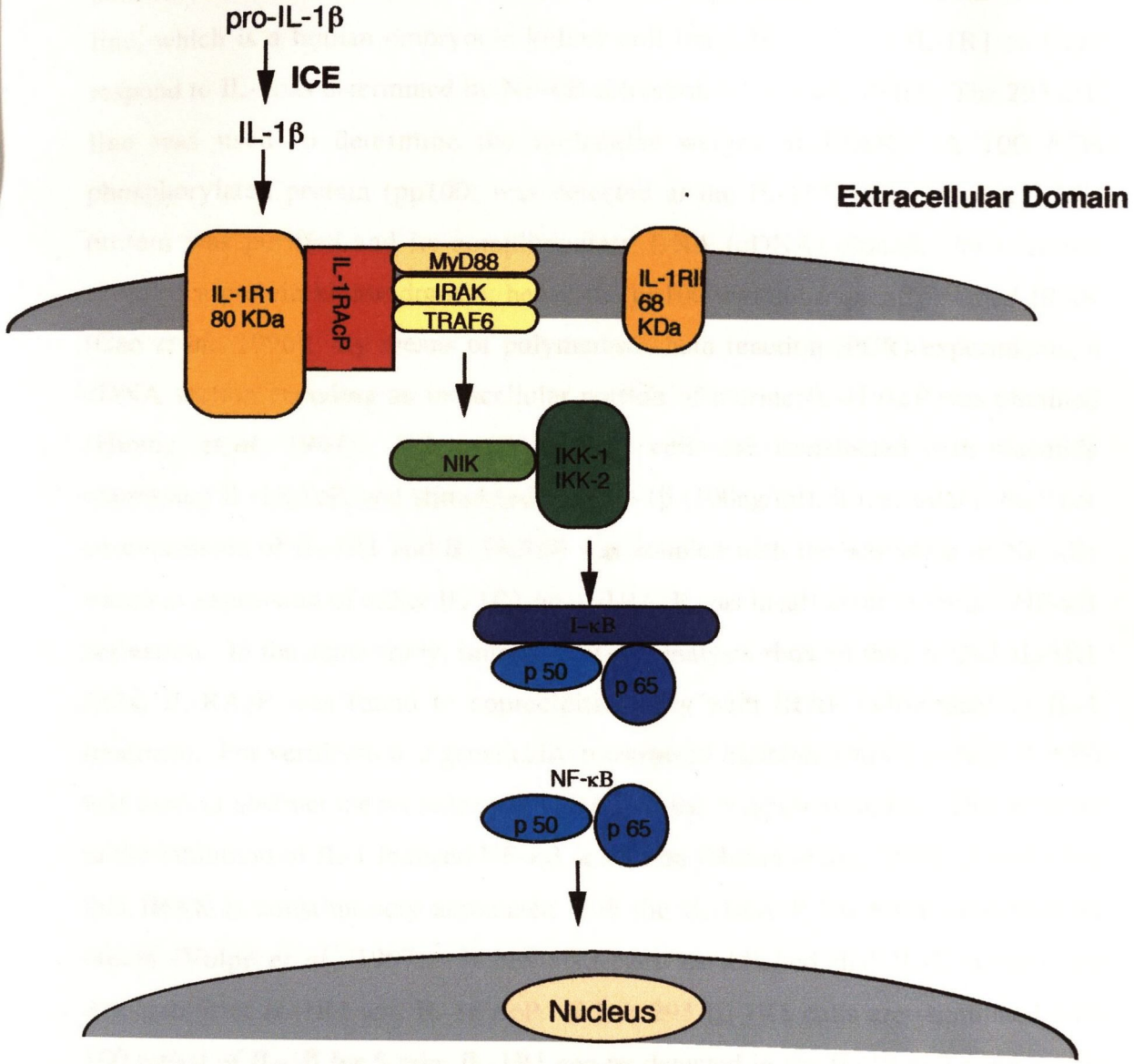
An IL-1-dependent kinase (IRAK) that associates with the IL-1R1 was identified in 1996 and has been shown to play a pivotal role in IL-1 signal transduction (Cao *et al.*, 1996). It is believed that IL-1 exerts its effects by inducing the expression of genes whereby their activity is tightly regulated by transcription factors such as nuclear factor-κB (NF-κB) and activating protein-1 (AP-1; see Stylianou & Saklatvala, 1998). The role for IRAK appears to be related only to the NF-κB and stress-activated protein kinase (SAPK), JNK and p38 signalling pathways. The following sequence of events is believed to occur (see fig 1.4). When IL-1 binds to IL-1R1, the IL-1 receptor accessory protein (IL-1RAcP) forms a complex with IL-1R1. It has been established that IL-1AcP is critical in the initial stages of IL-1 signalling cascades (Wesche *et al.*, 1997). The next event in this cascade is the recruitment of IRAK to the receptor complex. An adapter protein, MyD88, facilitates this action by docking with the complex (Croston *et al.*, 1995; Burns *et al.*, 1998), and phosphorylation of IRAK follows, which subsequently causes an interaction with a member of the Tumour Necrosis factor (TNF) receptor-associated family (TRAF), TRAF 6 (Cao *et al.*, 1996).

TRAF 6 is then activated by IRAK, resulting in the activation of NF-κB-inducing kinase (NIK). It is known that NF-κB is retained within the cytoplasm by inhibitory-κB kinases known as IKK's. Two forms of these kinases exist, IKK-1 and IKK-2 (Begg *et al.*, 1993; DiDonato *et al.*, 1997). Once NIK becomes phosphorylated, IKK activation occurs, leading to the phosphorlyation of I-κB. The phosphorylated form of I-κB degrades by proteosomes via a ubiquitin pathway which initiates the activation of NF-κB by freeing its two components, p65 and p50. This

Fig 1.4 The NF-κB signalling pathway:

The NF-κB cell signalling pathway is activated by an appropriate stimulus which induces receptor-mediated activity of IL-1β. A series of events transpire which involve the recruitment of IRAK, the MyD88 adaptor protein and TRAF6. Following these events, the inhibitory-κB kinases become activated and then degraded by proteosomes, thus initiating the activity of NF-κB by freeing its components p65 and p50. These signalling events lead to changes in gene transcription.

Adapted from "Potential mechanisms of IL-1 involved in cerebral ischaemia". (1999) Touzani, O. Boutin, H., Chuquet, J and Rothwell, N. *J. Immunol.* **100** 203-215.


pro-IL-1β
ICE
IL-1β
Extracellular Domain
IL-1R1
80 KDa
IL-1RAcP
MyD88
IRAK
TRAF6
IL-1RI
68
KDa
NIK
IKK-1
IKK-2
I-κB
p 50
p 65
NF-κB
p 50
p 65
Nucleus

final signalling event leads to translocation of free NF-κB through nuclear pores allowing binding to nuclear DNA and gene transcription (see Dinarello, 1999; Touzani *et al.*, 1999)

The NF-κB pathway has been extensively researched by various groups trying to determine the precise roles that IRAK, MyD88, IL-1RAcP and IL-1R1 play in this pathway. Most commonly used in these types of experiments is the 293 IL-1R1 cell line, which is a human embryonic kidney cell line. It expresses IL-1R1 and can respond to IL-1, as determined by NF-κB activation (Cao *et al.,* 1996). The 293 cell line was used to determine the molecular weight of IRAK. A 100 KDa phosphorylated protein (pp100) was detected at the IL-1/IL-1R1 complex. This protein was purified and its complimentary DNA (cDNA) cloned. Its structure revealed a protein kinase domain, hence the pp100 was subsequently named IRAK (Cao *et al.*, 1996). By means of polymerase chain reaction (PCR) experiments, a cDNA section encoding an intracellular portion of murine IL-1RAcP was obtained (Huang *et al.,* 1997). When 293 IL-1R1 cells are transfected with plasmids expressing IL-1RAcP, and stimulated with IL-1β (100ng/ml), it was established that co-expression of IL-1R1 and IL-1RAcP was coupled with the activation of NF-κB; transient expression of either IL-1R1 or IL-1RAcP was insufficient to induce NF-κB activation. In the same study, immunoblotting analysis showed that, in 293 IL-1R1 cells, IL-RAcP was found to coprecipitate only with IRAK subsequent to IL-1 treatment. For verification, a genetically constructed inhibitor known as AcP (1-403) was used to obstruct the recruitment of IRAK to the receptor complex. This resulted in the inhibition of IL-1-induced NF-κB activation (Huang *et al.,* 1997). The finding that IRAK is constitutively associated with the IL-1RAcP has been confirmed by others (Volpe *et al.,* 1997). It has also been established that IL-1 induces the association of IL-1R1 and IL-1RAcP. When 293 IL-1R1 cells are stimulated with 100 ng/ml of IL-1β for 5 min, IL-1R1 can be detected in the IL-1RAcP complex; in untreated cells this does not occur (Huang *et al.,* 1997).

One of the downstream consequences of IL-1 signalling is activation of p38 and JNK. In order to establish the involvement of IRAK in this cascade of events, IRAK-deficient mice were generated by genetically modifying exon sequences of the mouse IRAK gene. Skin and embryonic fibroblast cells containing a disrupted IRAK gene were stimulated with IL-1β. In the IL-1-treated cells, it was shown that

activation of JNK, p38 and NF-κB was decreased significantly, but not completely abolished (Kanakaraj *et al.*, 1998). Similar experiments have shown that over-expression of IRAK leads to prolonged and enhanced IL-1-stimulation, inducing JNK and NF-κB activation in EL-4 cells (Knop *et al.*, 1998). It has been also reported that IRAK has the ability to autophosphorlyate on multiple residues, thus withdrawing from the receptor complex and consequently being degraded by proteolytic cleavage. This may be the mechanism by which IRAK terminates its signalling at the receptor complex. Thus it has been suggested that phosphorylated IRAK may restrict itself in the event of IL-1 signalling, acting as a protective mechanism for cells to avoid prolonged activation (Knop & Martin, 1999).

MyD88, which is involved is the recruitment of IRAK to the receptor complex following IL-1 stimulation, acts by binding to IRAK, causing it to bind to IL-1R1 (Muzio *et al.*, 1997; Wesche *et al.*, 1997). This myleoid differentiation protein has no known biological function (Lord *et al.*, 1990), but may have certain signalling abilities due to its structural formation. MyD88 is organized into two domains, an N-terminal death domain (DD) which is similar to those found in the TNF-receptor family, and a C-terminal Toll domain, similarly found in the expanding IL-1R family (Feinstein *et al.*, 1995; Burns *et al.*, 1996). Dominant negative mutants of MyD88, called MyD88-lpr and MyD88-Toll, with disrupted DD and Toll domains respectively, enable the functions of these domains to be elucidated. It was found that the MyD88-DD is essential for the MyD88-activation of NF-κB and JNK, and the MyD88-lpr caused an inhibitory effect, suggesting that the DD acts downstream in this signalling cascade. It has been established that MyD88 operates by using its dual domain complex as an adapter which links Toll-and DD-containing proteins in IL-1 signal transduction (Burns *et al.*, 1996).

Homologues of IRAK have been identified; for example, a kinase similar in sequence and function to IRAK was discovered and designated IRAK-2. Using mutated forms of IRAK and IRAK-2, it has been determined that MyD88 acts upstream of IRAK-2 (Muzio *et al.*, 1997). Additionally, an IRAK-like molecule appointed IRAK-M has been discovered, primarily in monomyeloic cells. By using 293 IL-1R1 cell lines, it was found that overexpression of IRAK-M caused NF-κB activation. The activity of IRAK-M was shown to be similar to that of IRAK-2, but not as potent as IRAK itself (Wesche *et al.*, 1999). To further characterize this

kinase, transfection experiments showed that IRAK-M interacts with MyD88 and TRAF6. This suggests that IRAK-M plays a similar role in IL-1signal transduction to that of IRAK-2 and IRAK (Wesche *et al.*, 1999). I

It has been established that IL-1 and IL-18 share similar signalling pathways especially in activating the NF-κB pathway (Kojioma *et al.*, 1998), even though IL-18 is more potent than IL-1(Hunter *et al.*, 1997). Although IL-18 stimulates the activation of NF-κB resulting in its transloction to the nucleus, there has been no evidence to suggest that IL-18 is involved in the activation of p38 (see Dinarello, 1999). Because IRAK plays a pivotal role in IL-1 signalling, many reports suggest that the use of inhibitors directed towards IRAK may be extremely beneficial therapeutically for the treatment of IL-1-induced inflammatory diseases (Kanakaraj *et al.*, 1998).

1.7 Apoptosis

1.7.1 Evidence for apoptosis

In 1972 the term apoptosis was originally introduced and described (Kerr *et al.,* 1972) as a the ability of a cell to commit suicide, a process that is fundamental in a variety of biological systems such as normal cell turnover, the immune system, embryonic development, metamorphosis and in chemical-induced cell death. However, inappropriate cell death is believed to be implicated in various neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, ischemic damage, autoimmune diseases and various types of cancer (see Cohen, 1997). Distinctive morphological and biochemical changes take place during apoptosis and these lead to a controlled breakdown of the cell into fragments known as apoptotic bodies, which are consequently engulfed by macrophages. Typical morphological markers of apoptosis are DNA fragmentation and condensation, the compaction of cytoplasmic organelles and a decrease in cell volume and membrane blebbing, which result in the recognition and phagocytosis of apoptotic cells (Arends & Wyllie, 1991).

Initial findings were demonstrated in the nematode *Caenorhabditis elegans* . It was established that during the course of its normal development precisely 131 cells out of 1090 undergo apoptotic cell death (Ellis *et al.,* 1991). Later, studies showed that three essential genes were involved in this process, namely, *ced-3* and *ced-4,* both

of which were both found to be pro-apoptotic, and the *ced-9* gene which was found to be anti-apoptotic (Hengarter & Horvtiz, 1994). It was on the basis of identification of these genes in *C. elegans* that their mammalian homologues were identified, namely ICE/*ced-3*, Apaf/*ced-4* and Bcl-2/*ced-9* (Budihardjo *et al.*, 1999). These initial findings allowed characterization of the family of caspases, which orchestrate the cell death pathway.

1.7.2.Caspases

At the heart of the apoptotic pathway is the family of cysteine proteases known as caspases, which are related to the mammalian interleukin-1β-converting enzyme (ICE)/caspase-1 and the ced-9 gene (Nicholson & Thornberry, 1997). The family of caspases comprises of 14 cysteine protease that cleave proteins at aspartate residues upon activation. It is known that caspases are synthesised as pro-enzymes which are activated upon cleavage at specific sites. This group of proteases can be divided into subfamilies; the ICE subfamily (caspases 1, 4, 5, 11, 12, 13 and 14), the ced-3 subfamily (caspases 2, 3, 6, 7, 8, 9, and 10; Marks & Berg, 1999).

For the purposes of this thesis, only ICE and caspase-3 will be discussed. ICE was one of the first caspases to be discovered and is primarily responsible for the proteolytic conversion of the 31kDa inactive cytokine precursor, pro-IL-1β, to its mature and active 17kDa form (Thornberry *et al.*, 1992). [The biological activity of ICE has been discussed in section 1.1.5.]. Caspase-3 is regarded as one of the key executioners of the apoptotic pathway, being responsible either partially or completely for the proteolytic cleavage of many key enzymes, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). This caspase was initially identified, cloned and shown to encode a 32 kDa cysteine protease, CPP32 (Nicholson *et al.*, 1995).

The use of caspase-3 knockout mice has allowed assessment of the efficacy of caspase-3 in neuronal apoptosis. Kuida and colleagues (1996) reported that these mice were smaller in size and died after 1-3 weeks of age. The overall brain mass was markedly larger than that of wild type, cell development was disorganized and ectopic masses in the cortex, hippocampus and cerebellum were evident. It was also observed that these defects did not extend to other tissues and organs, thus suggesting that caspase-3 plays a dominant and non-redundant role in neurogenic apoptosis

(Kuida *et al.*, 1996). Caspase-3 is activated in neurons following spinal cord injury (Li *et al.*, 2000) in the human brain following head injury (Clark *et al.*, 1999) and in endotoxin-induced apoptosis in Kupffer cells (Hamada *et al.*, 1999).

It has been observed that caspases sequentially activate other caspases, establishing a hierarchy. Caspase-8 has been termed an "initiator" protease, which functions to amplify activity of caspases such as ICE, which subsequently activates caspase-3 or caspase-7. This hierarchical arrangement is further confirmed by the fact that caspases that cleave PARP (capase 3 and 7) take precedence over those that cleave lamins (caspase 6), therefore activation of caspase 3 and 7 is prior to activation of caspase-6 (see Cohen, 1997).

1.8 Lipopolysaccharide (LPS)

1.8.1 The activity of LPS is receptor-mediated

LPS is an integral cell wall component of Gram-negative bacteria that is crucial for bacterial survival. The critical components of LPS that are obligatory for the growth of Gram-negative bacterial are the lipid A moiety and two 3-deoxy-D-manno-octulosonic acid residues. Following lysis of the bacteria by antibiotic agents or host immune modulators, LPS becomes more accessible to the host's circulation and induces target cells, namely macrophages and monocytes, to release inflammatory mediators that initiate a cascade of soluble mediators (see Marsh & Wewers, 1996). LPS has been instrumental in cytokine biology due to its potency and ability to modulate cell activity. A large volume of experimental research has shown that LPS mediates its effects through second messengers, including cytokines (see section 1.8.2).

LPS interacts with target cells through binding to CD14, a 55kDa glycoprotein that exists as a glycosylphosphatidylinositol-anchored protein on the surface of monocytes and neutrophils (Wright *et al.*, 1990). It has been generally accepted that CD14 binds to the lipid A moiety of the LPS complex, a reaction that is catalyzed by an LPS-binding protein (LBP; Haliman *et al.*, 1995). LBP is a 60 kDa glycoprotein, synthesized by liver hepatocytes and functions by binding to the lipid A region of LPS (Henderson *et al.*, 1996). Until recently it was unknown what signalling receptor was involved in the receptor-mediated activation of LPS (O'Neill & Dinarello, 2000). However, just recently it was discovered that LPS utilizes a receptor known as toll-

like receptor-4 (TLR-4), while the TLR-2 is utilized by Gram-positive bacteria (Yoshimura *et al.,* 1999). These receptors two members of the IL-1 receptor/toll-like receptor superfamily (Rock *et al.,* 1998) which were identified on the basis closely linked homology *Drosophila* toll. The discovery that LPS mediates its action through TLR-4 was initially discovered in C3H/HeJ and C567Bl/10ScCr mice with a mutated TLR-4 gene, where LPS signalling was defective (Poltorak *et al.,* 1998). It has been proposed that additional adaptors are necessary for LPS action and one candidate is likely to be the MyD88 adaptor protein, since LPS-induced JNK and NF-κB activation is absent in MyD88-deficient mice (Kawai *et al.,* 1999). It has been proposed by Swantek and colleagues (2000) that LPS activation transpires in the following manner; LPS is transferred from either LBP or soluble CD14 receptor to a membrane bound CD14 receptor, where TLR4 is activated, although the ligand for this receptor is still unknown. Upon TLR4 activation, the IL-1 receptor associated kinase (IRAK) is activated, leading to a downstream signalling cascade resulting in the activation of the stress-activated protein kinases of NF-κB.

1.8.2 LPS and IL-1β

Infection and inflammation are known to induce host-defence responses that include the acute-phase responses and symptoms of brain-mediated illness. Additionally, production and release of proinflammatory cytokines is a characteristic feature in triggering the acute-phase response. Many studies have attempted to mimic these effects through intraperitoneal administration of LPS, and a variety of evidence exists to suggest that LPS-induced IL-1β production is involved in the development of neurological manifestations and in neuroinflammatory/immunological responses to LPS.

It was demonstrated that IL-1β mRNA, IL-1RI mRNA, IL-1ra mRNA and TNF-α mRNA levels were all significantly increased in the hippocampus, cerebellum and hypothalamus of LPS-treated rats (Ilyin *et al.,* 1998). Rats treated with LPS are prone to neurological manifestations such as anorexia, fever and sleep changes (Ilyin *et al.,* 1998). These results support the concept that proinflammatory cytokines, particularly IL-1β mediates the action of LPS in the central nervous system. These findings were confirmed by Van Dam and colleagues (1998) who demonstrated that peripheral administration of LPS induces a time- and dose-dependent increase in

immunoreactivity of IL-1β in meningeal macrophages, perivascular and microglial cells, while IL-1α was expressed 8 hours later and to a lesser extent. It was also shown that IL-1ra was constitutively expressed in the paraventricular nucleus and supraoptic nucleus. From this study it was suggested that LPS-induced IL-1β in the brain plays a role in the induction of symptoms during peripheral infection or inflammation (Van Dam *et al.,* 1998). Quan and co-workers (1999) examined the effects of low and high doses of LPS on cytokine production in the rat brain. The results indicated that low doses (0.01-10μg/kg) of LPS, induced IL-1β and TNF-α mRNA expression only in the meninges, circumventricular organs and choroid plexus, while higher doses (>500μg/kg) of LPS induced widespread expression IL-1β and TNF-α mRNA, thus suggesting that induction of these cytokines is dose-dependent and is concentrated at the blood barrier and circumventricular organs (Quan *et al.,* 1999). Mice deficient in ICE, the enzyme necessary to cleave IL-1β into a mature and active form, have been used to determine the importance of IL-1β in the murine brain in response to LPS-induced anorexia (Yao *et al.,* 1999). The results from this study demonstrated that mice lacking ICE, were resistant to LPS-induced anorexia, while an ICE analogue restored the anorexic properties associated with LPS. The same study also observed a resistance to LPS-induced anorexia in control mice treated with an ICE inhibitor.

1.9 Objectives of this study:

The primary objectives of this study were:

- To assess the effect IL-1β on endogenous glutamate release *in vitro* and to establish whether the stress-activated protein kinases, JNK and p38 might play a role in IL-1β-induced changes
- To investigate the effect of IL-1β on LTP and determine whether or not there is a role for JNK and p38 in IL-1β-induced impairment of LTP.
- To investigate the hypothesis that an increase in ROS formation underlies the age- and IL-1β-associated changes in LTP by examining the effects of dietary supplementation with the anti-oxidants vitamin E and vitamin C.
- To investigate the effects of LPS, on IL-1β concentration in brain tissues.
- To examine the possibility that apoptotic events induced by LPS are associated with impaired synaptic function.

Chapter 2

Materials and Methods

2.1 Materials

The full names and addresses of the sources listed below are given in Appendix II

Materials	Source
Acetic Acid	Sigma
Acrylamide	Sigma
Anti-mouse IgG	Sigma
Anti-mouse IgM	Sigma
Anti-rabbit IgG	Amersham
Aprotinin	Sigma
Bio-Rad	Bio-Rad Laboratories
β-mercaptoethanol	Sigma
Bovine serum albumin (BSA)	Sigma
Bovine serum albumin (BSA), non-acetylated	Sigma
Bromophenol blue	Sigma
$CaCl_2$	Sigma
Caspase-1 Inhibitor (Ac-YVAD-CMK)	Calbiochem
Caspase-1 substratae (DEVD peptide)	Santa Cruz
Caspase-3 substrate (YVAD peptide)	Santa Cruz
Cellulose acetate strips	Sartorius
DeadEnd™Colorimetric Apoptosis Detection System	Promega
Dimethyl sulphoxide (DMSO)	Lennox
Dithiothreitiol (DTT)	Sigma
2'7' dichlorofluorescein	Sigma
2'7' dichlorofluorescin diacetate	Molecular probes
DNAse	Sigma
dL-α-tocopheryl Acetate (diet)	Beeline Healthcare
DuoSet ELISA IL-1β Kit	Genzyme
Enhanced chemiluminescence (ECL) detection kit	Amersham
Ethanol	Sigma
Ethanolamine	Sigma
Ethylenediamineteraacetic Acid (EDTA)	Sigma
Glycerol	Sigma

HEPES	Sigma
Hydorchloric Acid (HCl)	Sigma
L-Ascorbic Acid (Diet)	Beeline Healthcare
Leupeptin	Sigma
Lipopolysaccharide (LPS)	Sigma
Magnesium Sulfate	Sigma
Mercaptoethanol	Sigma
Microscope Glass Cover Slips (22 X 26mm)	Chance Proper Ltd.
Microcsope Slides (76 X 26mm)	Chance Proper Ltd.
Nuclear Factor-κB (NF-κB) oligonucleotide	Promega
Nitrocellulose membranes	Sartorius
Normal donkey serum	Sigma
Parformaldehyde	Agar Scientific Ltd.
PD 098095	Calbiochem
p-p38	Santa Cruz
p-JNK	Santa Cruz
Pepstatin	Sigma
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
POLY(dI-dC). POLY(dI-dC)	Amersham
Protease X	Sigma
Protease XIV	Sigma
SB 203580	Calbiochem
Sodium Dodecylsulfate (SDS)	Sigma
Sulfuric Acid	Sigma
3,3',5,5' - Tetramethyl-bezidine (TMB)	Sigma
T_4 Polynucleotide Kinase	Promega
Trition X-100	Sigma
Trypsin	Sigma
Tween-20	Sigma
Urethane	Sigma
Whatman filter paper	Whatman
All other basic chemicals	Sigma and Lennox

2.2 Animals

2.2.1 Housing of animals

Male Wistar rats were used in all experimental procedures. These rats were an inbred strain obtained from the BioResources Unit (BRU), Trinity College, Dublin and were 2 to 4 months old, weighing between 250g-300g. Animals were housed in groups of 6, unless otherwise stated.

In some experiments the effects of age were analysed. 22 and 24 months old rats, weighing between 500g and 550g were an inbred strain supplied by either the BRU of Trinity College or Charles River Laboratories, U.K. When aged rats were obtained from Charles River Laboratories were used, young rats from this source were also used. The aged rats were housed in pairs. All animals were maintained under a 12-hour light-dark cycle in the BRU. Ambient temperature was controlled between 22 and 23°C and food (normal laboratory chow) and water was available *ad libitum.*

2.2.2 Dietary manipulation

In one series of experiments the effects of dietary manipulation was assessed in aged and young rats. Animals were divided at random into subgroups. Subgroups of rats were fed an experimental diet (vitamin diet) of laboratory chow to which dL-α-tocopheryl acetate (vitamin E; 250mg/rat/day; Beeline Healthcare, Ireland) dissolved in corn oil was added. Rats were also given drinking water containing L-ascorbic acid (vitamin C; 250mg/rat/day; Beeline Healthcare, Ireland). Control groups were given standard laboratory chow to which corn oil was added, to ensure both subgroups received an isocaloric intake. Before starting the dietary manipulation, food and water intake were measured for 5 days.

In a separate experiment, the effect of a second diet was assessed. In this case aged and young rats were divided into two groups. One subgroup of young rats and one of aged rats were fed for 8 weeks on an experimental diet of laboratory chow supplemented with a daily dose of 10 mg docohosahexanoic acid, 26% w/v in tuna oil; Laxdale Research, U.K.). The other subgroups were given standard laboratory chow to which corn oil was added, to ensure both subgroups received an isocaloric intake.

2.3 Preparation of tissue

Animals were killed by cervical dislocation and decapitation and the brains were rapidly excised and placed on ice. Hippocampi, entorhinal cortices, or dentate

gyri (in the case of LTP experiments; control and experimental side; see below 4.2.3) were dissected free. This procedure took approximately 2 min.

2.3.1 Preparation of slices for freezing

Freshly dissected tissue, either dentate gyri, hippocampi, or enthorinal cortex, was sliced bidirectionally to a thickness of 350μm using a McIlwain tissue chopper and rinsed in ice-cold oxygenated Krebs solution (composition: NaCl, 136mM; KCl, 2.54mM; KH_2PO_4, 1.18mM; $Mg_2SO_4.7H_20$, 1.18mM;$NaHCO_3$, 16mM; Glucose, 10mM) containing $CaCl_2$ (final concentration: 2mM). These slices were allowed to settle and were rinsed again. Finally the slices were rinsed twice with ice-cold oxygenated Krebs solution containing $CaCl_2$ (2mM) and DMSO (final concentration: 10%) and then stored in this solution at -80°C until required for further analysis (Hans & Bowen, 1981). When required, slices were thawed rapidly at 37°C and washed three times with ice-cold oxygenated Krebs solution containing $CaCl_2$ (2mM).

2.3.2 Preparation of Synaptosomes

Synaptosomes were prepared either from freshly dissected tissue or, in other experiments, from frozen slices. In the cases of fresh tissue, synaptosomes were prepared in the following manner; the tissue was homogenized with 15 up and down strokes in 1ml ice-cold sucrose (final concentration: 0.32M) and the homogenate was centrifuged at 5,000rpm at 4°C for 5min. The supernatant was removed and centrifuged for 15,000rpm at 4°C for 15 min. The resulting pellet was P_2, an impure synaptosome-enriched preparation. When synaptosomes were prepared from frozen slices, slices were rapidly thawed at 37°C and rinsed three times with excess ice-cold Krebs solution containing $CaCl_2$ (2mM), then homogenized and spun as for fresh tissue to obtain P_2.

2.4 Protein quantitation using the Bradford assay

Calculation of protein in tissue was carried out according to the method of Bradford (1976). Standards were prepared from a stock solution of 200μg/ml of bovine serum albumin (BSA) to a volume of 160μl and ranged from 4μg/ml to 100μg/ml. Samples (5μl) were loaded onto a 96-well plate and then diluted with

distilled water (155µl). Bio-Rad dye reagent (40µl) was added to all preparations, which were then mixed and incubated at room temperature for 5 min. Absorbance was assessed at 630nm using a 96-well plate reader (EIA multiwell reader, Sigma). The concentration of protein in the tissue samples was calculated from the regression line plotted from the standard curve.

2.5 Induction of LTP *in vivo*

2.5.1 Preparation of animals

Animals were anaesthetized by an intraperitoneal (i.p.) injection of urethane (1.5g/kg) until loss of consciousness occurred, which was apparent by the absence of the pedal reflex. When necessary, a further top-up dose (to a maximum of 2g/kg) was given. The scalp fur was clipped off and the animals were placed in a sterotaxic frame. A midline incision was made and the periosteum was scraped clean to reveal the skull plates and allowing identification of lambda and bregma. A dental drill was used to remove a window of skull revealing the brain and the dura mater was carefully peeled away allowing the electrode implantation. The recording chamber consisted of a sterotaxic unit permanently attached to the bench to maintain stability and surrounded by a Faraday cage to isolate the signal from environmental interference. All instruments in the cage were grounded to eliminate 50Hz cycle noise.

2.5.2 Electrode implantation

Bipolar stimulating electrodes and unipolar recording electrodes were obtained from Clark Electromedical, UK. The stimulating electrode was placed on the surface of the brain, 4.4mm lateral to lambda and initially lowered to a depth of approximately 2mm. The recording electrode was placed on the surface of the brain, 2.5mm lateral and 3.9mm posterior to bregma and was also lowered to a depth of approximately 2mm. The positions of the electrodes were carefully monitored as they were lowered through the cortical and hippocampal layers to the perforant path and the granule layer of the dentate gyrus. This was carried out by generating a 0.1msec duration, 2msec delay, 4 V pulse through the stimulating electrode at a frequency of 0.1Hz. Evoked responses were picked up by the recording electrode and displayed on an Apple Macintosh computer (Performa 200). The stimulating electrode was lowered, in increments, using the coarse manipulator control into the perforant path. The recording

electrode was lowered into the dentate gyrus until the characteristic perforant path granule cell synaptic response was observed with the final positions of both the recording and stimulating electrode adjusted to give a response of approximately 1mV in amplitude. Stimuli were then delivered at 30sec intervals. The final electrode positions were approximately 2-2.5mm below the surface of the brain for the stimulating electrode and 4-5mm for the recording electrode.

2.5.3 EPSP recordings

The population field excitatory postsynaptic (field EPSP) was used as a measure of excitatory synaptic transmission in the hippocampus. EPSPs were achieved by passing a single square wave pulse of current at low frequency (0.033Hz, 0.1msec, 2msec delay) generated by a constant current isolation unit (IsoFlex, U.K.) to the bipolar stimulating electrode. The evoked response was transmitted via a preamplifier (DAM 50; Differential Amplifier; gain 75, World Precision Instruments, U.S.A.) with a broad band setting of 4 Hz to 6 kHz, to an analogue-to-digital converter (MacLab/2e, Analogue Digital Instruments). This was a digitized system linked to an Apple Macintosh computer (Performa 200) which interfaced with the converter via a specifically written software package (Scope, version 3.36) which was customized to control both the generation of the square wave pulses and recording of the evoked potentials. The field EPSPs were therefore displayed on-line and could be analysed at the time of recording or at a later date.

The slope of the EPSP was taken as the main indicator of excitatory synaptic transmission. After an initial period of stabilization, test shocks at 1/30sec were recorded for a 10 min control period to establish stable baseline recordings. This was followed by a delivery of 3 high frequency trains of stimuli (250Hz for 200msec) at 30sec intervals. Recording at test shock frequency then resumed for 40 min.

2.5.4 Treatment Regimes

In some experiments, rats received injections that were administered intrpeitoneally (i.p.), or intracerebroventricularly (i.c.v.), or both, 3 h or 10 min prior to electrophysiological recordings and the induction of LTP. In these experiments, preparation for i.c.v. injection involved drilling a hole in the skull 0.4mm posterior to

bregma and 0.2mm lateral to the midline at a depth of 3.5mm. Injections volumes were 5µl and were given using a Hamilton syringe. The following table illustrates the drugs used and the manner in which they were administered:

Drugs	Administration	Dose
1. Saline	i.c.v./i.p.	5µl (0.9%)
2. Interleukin-1β	i.c.v.	5µl (3.5ng/ml)
3. Lipopolysaccharide (LPS)	i.p.	1ml (100/500µg/kg))
4. SB 203580 (p38 inhibitor)	i.c.v.	10µl (50µM)
5. Interleukin-1β-converting enzyme (ICE) inhibitor: (Ac-YVAD-CMK)	i.c.v.	5µl (10pmol)

2.6 Analysis of endogenous glutamate release

The synaptosomal preparation P_2 was prepared as described (see 2.3.2) and resuspended in oxygenated Krebs solution containing $CaCl_2$ (2mM) which had previously been brought to the reaction temperature of 37°C. In some experiments synaptosomes were preincubated in the presence of IL-1β (final concentration: 10pg/ml, 100pg/ml 1ng/ml, 3.5ng/ml, 100ng/ml), the ERK inhibitor PD 098059 (10µM) and the p38 inhibitor SB 203580 (50µM, 100µM) or a combination of these drugs, for 20 min at 37°C. Samples were oxygenated continuously during this period. Aliquots of the pretreated synaptosomes (25µl) were pipetted onto a filtration manifold in which cellulose acetate strips (pore size 0.45µM) were placed. Samples were washed under vacuum at least 10 times with oxygenated Krebs solution (250µl) containing $CaCl_2$ (2mM) maintained at 37°C. Synaptosomes were incubated for 3 min at 37°C with either Krebs solution containing $CaCl_2$ (2mM) or Krebs solution containing both $CaCl_2$ (2mM) and KCl (final concentration: 50mM), to depolarize the synaptic plasma membrane. Filtrates were collected under vacuum and stored at -20°C for later analysis of glutamate concentration by immunoassay. An aliquot (50µl) was retained for analysis of protein concentration.

2.7 Analysis of glutamate concentration by immunoassay

Endogenous glutamate was measured in stored samples (see 2.6) according to the method of Ordronneau and colleagues (1991). 96-well plates (Sigma Techware U.K.) were coated with coating buffer (250µl; composition NaH_2PO_4, 100mM; glutaraldehyde, 0.5% v/v pH 4.5) and incubated for 1 h at 37°C, after which time plates were washed twice with NaH_2PO_4 buffer (250µl; 100mM, pH 8.0). Samples of filtrate (50µl) and glutamate standards (ranging from 12.5 nM to 500nM) were added to the coated wells. Plates were covered and incubated for 2 h at 37°C and then washed 4 times with Na_2HPO_4 buffer. Non-specific binding was blocked in 2 steps; firstly for 1 h at 37°C with ethanolamine (250µl; 0.1M in Na_2HPO_4 buffer) and then for 1 h at 37°C with normal donkey serum (250µl; 3% v/v in Phosphate buffered saline-Tween (PBS-T; Na_2HPO_4, 80mM; NaH_2PO_4, 20mM; NaCl, 100mM; 0.05% Tween, pH7.3)). Plates were washed after each step with PBS-T.

Anti-glutamate antibody (Sigma, U.K.), the primary antibody, was diluted 1:5,000 with normal donkey serum (3% v/v in PBS-T) and an aliquot (100µl) of this solution was added to each well. To assess the background absorbance, normal donkey serum (100µl; 3% v/v in PBS-T) was added in the absence of anti-glutamate antibody. Plates were covered, incubated overnight at 4°C and washed 4 times with PBS-T. The secondary antibody (100µl) was HRP-linked anti-rabbit IgG (Amersham), diluted 1:10,000 with normal donkey serum (3% v/v in PBS-T) and was added to the plates and incubated for 1 h at room temperature. Plates were washed with PBS-T, tetramethylbenzidine (TMB) liquid substrate (100µl; Sigma, UK) solution was added to each well and the reaction was stopped by the addition of H_2SO_4 (50µl; 4M). The plates were agitated for 15 sec and the absorbances were read at 450nm using a 96-well plate reader. The concentration of glutamate in the samples was calculated with reference to the standard curve and the concentration of glutamate was expressed as µmol glutamate/mg protein.

2.8 SDS-PAGE

2.8.1 Preparation of tissue for the analysis of protein kinase activity:

The synaptosomal preparation P_2 was prepared as described (see 2.3.2). The P_2 pellet was resuspended in oxygenated Krebs solution containing $CaCl_2$ (2mM; KrebsCa) which had previously been brought to the reaction temperature of 37°C. In

some experiments, synaptosomes were preincubated in the presence of IL-1β (final concentration: 10pg/ml, 100pg/ml 1ng/ml, 3.5ng/ml and 100ng/ml) for 20 min at 37°C. In control experiments, synaptosomes were preincubated in KrebsCa solution. Samples were oxygenated continuously during this incubation period. The samples were equalized for protein concentration as previously described (see 2.4), sample buffer was added (10µl: composition: Tris-HCl pH 6.8, 0.5M; SDS, 10% w/v; glycerol, 10% v/v; 2-β-mercaptoethanol, 5% v/v; bromophenol blue, 0.05% w/v) and samples were boiled for 5 min and used immediately or stored at -80°C until further use.

2.8.2 Gel electrophoresis

10% and 12% acrylamide gels (see appendix II) were cast between 2 glass plates and affixed to the electrophoresis unit (Sigma Techware UK) using spring clamps. Electrode running buffer (composition: Tris base, 25mM; glycine, 200mM; SDS, 17mM; pH 7.4) was added to upper and lower reservoirs. The samples were loaded onto the gel. Appropriate separation of proteins was achieved by the application of a 30mA current until the bromophenol blue reached the bottom of the gel; this process took approximately 40 min.

2.8.3 Western Immunoblotting

The gel slab was removed from the glass plates and gently washed in transfer buffer (composition: Tris base, 25mM; glycine, 200mM; methanol, 20% (v/v); SDS, 0.5% (w/v) pH 8.3), for 5 min to remove any excess particles that may have adhered to the surface of the gel. One sheet of nitrocellulose paper (Sartorius, U.K.) and two sheets of filter paper (Whatman No. 3) pre-cut to the size of the gel were pre-soaked in transfer buffer for 5 min. A layered sandwich was made in which the nitrocellulose paper was placed on top of the filter paper. The gel slab was placed on top and covered by the second sheet of filter paper. Air bubbles were removed and the sandwich (pre-soaked in transfer buffer) was placed on the graphite electrode (the anode) of a semi-dry blotter (Biometra, U.K.). The lid containing the cathode was placed firmly on top. The transfer was carried out at 225mA for 75 min. Non-specfic binding was inhibited by incubating the blots overnight at 4°C with a solution of PBS-T (25ml) containing non-fat dry milk (8% w/v). The membrane was washed with PBS-T, with several changes between washes. The membranes were incubated for 2 h with the appropriate

primary antibodies, ERK (5ml; Promega; diluted 1:2,000 with 2% non-fat dry milk in PBS-T), JNK (5ml; Santa Cruz; diluted 1:1,000 with 2% non-fat dry milk in PBS-T), and p38 (5ml; Santa Cruz, diluted 1:1,000 with 2% non-fat dry milk in PBS-T). These antibodies are specific for phosphorylated ERK, JNK and p38. Membranes were washed with PBS-T (approximately 15ml) for 1 h at room temperature, with several intermediate changes of buffer. The membranes were incubated with the appropriate secondary antibodies (for ERK; 5ml; anti-rabbitt IgG, Amersham; diluted 1:10,000 with 2% non-fat dry milk in PBS-T; for p38; 5ml; anti-mouse IgM; Sigma; diluted 1:1,000 with 2% non-fat dry milk in PBS-T; for JNK; 5ml; anti-mouse IgG; Sigma; diluted 1:1,000 with 2% non-fat dry milk in PBS-T) for 2 h at room temperature, followed by several washes with PBS-T. Finally, both membranes were incubated for 1 min with Enhanced Chemiluminescence (ECL) detection reagent (2ml). Excess reagent was drained from the membranes and placed protein side up between two plastic acetate sheets before being exposed overnight at 4°C to photographic film (Hyperfilm, Amersham). The film was developed using a Fuji X-ray processor. Densitometric analysis of the bands was made using the Gelworks computer programme.

2.9 Analysis of IL-1β concentration using ELISA

For analysis of IL-1β concentration, the DuoSet ELISA (Enzyme Linked ImmunoSorbent Assay) development system for mouse IL-1β was used (Genzyme Diagnostics, USA). Although this kit detects mouse IL-1β, specific immunoreactivity against rat IL-1β was detected. Ninety-six well plates were coated with capture antibody (monoclonal hamster anti-mouse IL-1β; 100µl; 2.0µg/ml final concentration) diluted in sodium carbonate buffer (0.1M; pH 9.5; composition: 0.32g Na_2CO_3, 0.58g $NaHCO_3$ in 100ml distilled H_2O) and incubated overnight at 4°C. Plates were washed four times with PBS-T and blocked for 2 h at 37°C with blocking buffer (PBS with 4% (w/v) BSA). Blocking buffer was aspirated and aliquots (100µl) of samples (equalized for protein), and standards (range 0-1000pg/ml) were added to each well and incubated for 1 h at 37°C. Plates were washed four times with PBS-T. The secondary antibody (biotinylated polyclonal rabbit anti-mouse IL-1β; 100µl; 0.8µg/ml final concentration in PBS-T with 1% (w/v) BSA) was added to each well and incubated for 1 h at 37°C. The plates were washed four times and a detection reagent (horseradish-peroxidase

conjugated streptavidin; 100µl; 1:1,000 dilution in PBS-T with 1% (w/v) BSA) was added to each well and incubated for a period of 15 min at 37°C. The plates were washed again four times with PBS-T. Aliquots of substrate (tetramethylbenzidine (TMB) liquid substrate; 100µl, Sigma UK) were added to the wells and the plate was incubated for up to 20 min at room temperature, this reaction created a colour change to blue. The reaction was stopped by the addition of a "stop solution" (2N H_2SO_4; 100µl). The absorbance values were determined by reading the plate at 450nm within 30 min. IL-1β concentration in samples was established from a standard curve constructed by plotting the mean absorbance for each standard versus the corresponding concentration. Aliquots of tissue was stored at -20°C for later analysis of protein content (Bradford, 1976) and the IL-1β concentration was expressed as equivalent of pg IL-1β/mg protein.

Due to a change over in manufacturers of the IL-1β DuoSet ELISA kit from Genzyme diagnostics to R & D Systems, the protocol for the IL-1β ELISA changed slightly during the course of this study. Briefly, 96 well plates were coated with capture antibody (monoclonal hamster anti-mouse IL-1β; 100µl; 4.0µg/ml final concentration) diluted in sodium carbonate buffer (0.1M; pH 9.5; composition: 0.32g Na_2CO_3, 0.58g $NaHCO_3$ in 100mls distilled H_2O) and incubated overnight at 4°C. Plates were washed four times with PBS-T and blocked for 2 h at RT with blocking buffer (PBS with 1% (w/v) BSA). Blocking buffer was aspirated and aliquots (100µl) of samples (equalized for protein), and standards (range 0-1000pg/ml) were added to each well and incubated for 2 h at 37°C. Plates were washed four times with PBS-T. The secondary antibody (biotinylated polyclonal rabbit anti-mouse IL-1β; 100µl; 100ng/ml final concentration in PBS-T with 1% (w/v) BSA) was added to each well and incubated for 2 h at room temperature (RT). The remainder of the protocol was followed as described above.

2.10 Analysis of Interleukin1β Converting Enzyme (ICE) activity

Slices of hippocampus stored in Krebs/DMSO were thawed and washed three times in fresh Krebs solution. The slices were incubated on ice in lysis buffer (400µl; 25mM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml leupeptin, 10µg/ml pepstatin) for 30 min and then homogenised in the lysis buffer. Samples were centrifuged at 15,000rpm at 4°C for 20 min. The supernatant was

removed (90µl) and added to 500µM ICE substrate (10µl) (YVAD peptide, Santa Cruz) and the solution was incubated for 1h at 37°C. Incubation buffer (900µl; 100mM HEPES, 5mM DTT, pH 7.4) was added to the solution and the samples were transferred to cuvettes. Emission at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25µM to 100µM) were prepared and a standard curve of absorbance against concentration was plotted. Results were expressed as nmol AFC/mg protein/min.

2.11 Analysis of Caspase–3 activity

Slices of hippocampus stored in Krebs/DMSO were thawed and washed three times in fresh Krebs solution. The slices were incubated on ice in Lysis buffer (400µl; 25mM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml leupeptin, 10µg/ml pepstatin) for 30 min and then homogenised in the lysis buffer. Samples were centrifuged at 15,000rpm at 4°C for 20 min. The supernatant was removed (90µl) and added to 100µM caspase-3 substrate (10µl; DEVD peptide, Santa Cruz, U.S.A) and the solution was incubated for 1h at 37°C. Incubation buffer (900µl; 100mM HEPES, 5mM DTT, pH 7.4) was added to the solution and the samples were transferred to cuvettes. Emission at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25µM to 100µM) were prepared and a standard curve of absorbance against concentration was plotted. Results were expressed as nmol AFC/mg protein/min.

2.12 Analysis of reactive oxygen species (ROS)

Slices of hippocampus stored in Krebs/DMSO were prepared as described in section 2.3.1. and synaptosomes which were used for analysis of ROS were prepared as described in detail in section 2.3.2. Ice-cold Tris buffer (1ml; 40mM, pH 7.4) was added to the remaining synaptosomal pellet, followed by DCFH-DA probe (10µl, 5µM, Molecular probes, Netherlands). The samples were incubated for 15 min at 37°C. In order to terminate the reaction, the dye-loaded synaptosomes were centrifuged at 13,000 rpm at 4°C for 8 min. The pellet was resuspended in 1.5ml of ice-cold Tris buffer (40mM; pH 7.8). Fluorescence was monitored at 488nm excitation (band width: 5nm) and 525nm emission (band width: 20nm) in a fluorescent spectrometer linked to a Macintosh Performa 5200 via a MacLab 2E Data

Acquisition System. Protein concentration was assessed as previously described in section 2.4. and results were expressed as nmol DFC/mg protein.

2.13 Preparation of hippocampal and entorhinal cortical neuronal cells

2.13.1 Dissociation of cells

Slices (350μm) prepared from entorhinal cortex and hippocampus were equilibrated in oxygenated Krebs solution for 30 min at 30°C and then incubated in Krebs solution containing protease X (1mg/ml), protease XIV (1mg/ml) and DNAse (1600 Kunitz) for 30 min at 30°C. Washed slices were resuspended in 1ml Dulbecco's modified essential medium containing DNAse (1600 Kunitz), triturated with a glass pasteur pipette and passed through a nylon mesh filter to remove tissue clumps. Suspensions of dissociated cells were cytospun (Shandon cytospin II) at 600rpm for 2 min to facilitate adherence onto glass microscope slides. The slide-mounted cells were immersed gently into methanol in order to fix the cells and were stored at RT, in the dark until required for analysis. In some experiments, e.g., for the analysis of transcription factors, the suspensions of dissociated cells were used.

2.13.2 DeadEnd™ Colorimetric Apoptosis Dectection System: TUNEL Assay

This system end-labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labelling) assay. Cytospun cells were prepared as described earlier (see section 2.13.1) circled with a pap pen and fixed with 4% paraformaldehye for 30 min at RT by placing a drop of the solution on top of the cells. The cells were washed by incubating with PBS (pH 7.4; 137mM NaCl, 8.1mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.68mM KCl) for 5 min. This procedure was repeated twice. The cells were permeabilized with PBS-Triton (1μl Triton: 1ml PBS; 1:500 dilution), and washed three times with PBS. Excess liquid was removed gently from the slide, cells were covered with 50μl of equilibration buffer (200mM potassium cacodylate (pH 6.6 at 25°C), 25mM Tris-HCl (pH 6.6 at 25°C), 0.2mM DTT, 0.25mg/ml BSA, 2.5mM cobalt chloride) and equilibrated for 5 min at RT. The cells were incubated with 30μl of Terminal deoxynucleotidyl transferase reaction mixture (TdT; 98μl equilibration buffer, 1μl biotinylated nucleotide mix, 1μl TdT enzyme) at 37°C for 1h. and slides were covered to ensure the cells did not dry out. The reaction was terminated by covering the cells with 100μl 2XSCC (1:10; 2XSCC:

deionized water) for 15 min at RT. Cells were washed with PBS three times, endogenous peroxidases were blocked with 100µl 0.3% H_2O_2 for 5 min at RT. and cells were washed again with PBS three times. Streptavidin HRP solution diluted in PBS (100µl; 1: 500), was added to the cells, incubation continued at RT for 30 min, to allow binding to the biotinylated nucleotides. The slides were washed in PBS three times. A diaminobenzidine (DAB) solution was used to stain apoptotic nuclei brown, doing so by binding to the streptavidin HRP. To each slide, 40µl of the DAB solution (final concentration: 1ml; 50µl DAB substrate 20X buffer, 50µl DAB 20X chromogen, 50µl H_2O_2 20X, 950µl deionized water) was added to the cells for a 10 min incubation period. The cells were washed several times with deionized water. The slides were mounted with glycerine jelly (10g gelatine; 60ml distilled H_2O; 70ml glycerine; 0.25g phenol). The cells were counted under a light microscope and positively stained cell numbers were noted. The results were expressed as % degenerative cells.

2.13.2 p38 staining in entorhinal cortical cells

Cell extracts were prepared from entorhinal cortex as described in section 2.13.1. Cells were washed in saline (0.9%; 100µl-for all incubation volumes) for 5 min at RT and fixed with paraformaldehyde solution (4% w/v in PBS; pH 7.4) for 30 min at RT before being washed twice in PBS and permeabilized with Triton-X100 (0.1% v/v in PBS) for 5 min at RT. Cells were refixed in paraformaldehyde solution (4% w/v in PBS) for 15 at RT, washed and incubated in H_2O_2 (0.6% in PBS) for 15 min at RT to block any endogenous peroxidases. Slides were washed in PBS, blocked in normal horse serum (1:20 v/v in PBS) for 30 min at RT and incubated in a phosphospecific anti-p38 primary antibody (1:100 in PBS; Santa Cruz, U.S.A.) for 2 h at 37°C. Cells were washed in PBS, incubated in biotinylated horse anti-mouse antibody (1:100; Vector; U.K.) for 30 min at RT and reacted with vectastin avidin biotin system for 15 min at RT to amplify the signal. Cells were incubated in Diaminobenzidine (DAB) chromagen solution containing H_2O_2 (0.1%) for 10 min at RT, rinsed in distilled and counterstained in methyl green. Cells were dehydrated through alcohol to xylene and mounted in DPX. Cells were counted and positive p38 cells were expressed as a percentage of the total number of cells counted.

2.14 Electrophoretic mobility band shift assay

2.14.1 Preparation of nuclear extracts from dissociated cells

Cell extracts were prepared from entorhinal cortex as described in section 2.13.1. Cells were centrifuged at 1,000 rpm for 1 min, resuspended in buffer A (1ml: 10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.50mM PMSF, and 0.5mM DTT; pH 7.9) and centrifuged at 15,000 rpm at 4°C for 5 min. The resulting pellet was resuspended in 20µl Buffer A containing NP40 (0.1%), placed on ice for 10 min and centrifuged at 15,000 rpm at 4°C for 10 min. The pellet was resuspended in Buffer C (15µl: 20mM HEPES, 20mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol (v/v), 0.5mM PMSF; pH 7.9), placed on ice for 30 min and centrifuged 15,000 rpm at 4°C for 10 min. Buffer D (40µl) was added to the supernatant (10mM HEPES, 50mM KCl, 0.2mM EDTA, 20% (v/v) glycerol, 0.5mM PMSF, 0.5mM DTT; pH 7.9), which was frozen at -80°C until further use.

2.14.2 Detection of NF-κB in nuclear extracts

The nuclear extracts were equalized for protein as described in section 2.4. The labelling reaction mixture was prepared (final volume: 2.85µl DNA; 10pmol, 2.5µl kinase buffer, 7.5µl ATP; 50pmol, 2.5µl T_4 kinase; 20 units, 9.65µl distilled H_2O). The labelling reaction mixture was placed in a perspex box pr-warmed to 37°C and incubated at 37°C for 10 min. The reaction was terminated by adding 2µl EDTA (0.5M; pH 8.0) to reaction mixture and 1 volume phenol:chloroform:isoamylalcohol. Meanwhile the [^{32}P] labelled DNA fragment containing the NF-κB motif was thawed out and counted. The radioactivity of the [^{32}P] labelled DNA probe was measured using a Packard 1500 Scintillation Spectrometer. The scintillation fluid used was Cocktail-T (Lennox, U.K.) to determine the radioactivity of the probe. Once the counts were determined, the probe was diluted with TE buffer to attain 20,000 counts per minute (cpm). The binding reaction mixture was prepared as follows: 10X binding reaction buffer (1:10 dilution; 40% glycerol, 10mM EDTA, 50mM DTT, 100mM Tris; pH 7.5, 1M NaCl, 1mg/ml nuclease free BSA; Sigma), 1µl [^{32}P] labelled DNA fragment, 15µl nuclear extract, 2µl non-specific competitor DNA (POLY(di-dC).POLY(di-dC); Amersham). The binding reaction mixture was incubated at RT for 30 min. Following the incubation period, sample buffer was added (1:10 dilution) to the samples. 4% acrylamide gels were prepared (see appendix II). Band shift

running buffer was prepared (4.5g sodium acetate, 67ml 1M Tris pH 7.5, 20ml 0.5M EDTA, 35µl mercaptoethanol in 1l) and added to the upper and lower resevoirs of the electrophoresis unit. The gel slab was affixed using spring clamps and the samples prepared were loaded into the wells and run at RT at 160 V for approximately 1 h. The gel was then dried onto filter paper under vacuum at 80°C for 30 min. The gel was exposed to film for one week at -80°C and processed using a Fuji X-ray developer.

Chapter 3

The analysis of the effects of interleukin-1β and stress-activated protein kinases on endogenous glutamate release in vitro

Chapter 3

The analysis of the effects of interleukin-1β and stress-activated protein kinases on endogenous glutamate release in vitro.

3.1 Introduction:

The proinflammatory cytokine, interleukin-1β (IL-1β) acts as a mediator of host defences in response to tissue injury and infection (see Dinarello, 1996). IL-1β is produced not only by immune cells, but also by neuronal and glial cells (Yao *et al.,* 1992) and can induce development and differentiation of these cell types (Rothwell *et al.,* 1996). Radioligand binding and *in situ* hybridization studies have localized IL-1 receptors in neuronal tissue and high densities have been observed in certain areas such as the granular and pyramidal cell layers of the hippocampus (Ban *et al.*, 1991).

Evidence to date suggests that IL-1 is a modulator of neuronal and synaptic plasticity and this concept has been explored using long-term potentiation (LTP) as a model for synaptic plasticity (Bellinger *et al.,* 1993). LTP is a putative animal model for learning and memory (Bliss & Lomo, 1973); it is a sustained increase in synaptic transmission and postsynaptic neuronal activity after high frequency stimulation of afferent fibers. LTP has been identified at synapses in several brain areas, but has been extensively studied in the hippocampus (Bliss & Collingridge, 1993). IL-1β has been shown to inhibit LTP *in vitro*, in mossy fiber-CA3 pathway of the mouse hippocampus (Katsuki *et al.,* 1990), in the rat dentate gyrus (Cunningham *et al.,* 1996) and in the CA1 region of the rat hippocampus (Bellinger *et al.,* 1993). The inhibitory effect of IL-1β on LTP in the rat dentate gyrus has also been shown *in vivo* (Murray & Lynch, 1998).

One of the biochemical changes associated with the induction of LTP is an increase in endogenous glutamate release in the dentate gyrus (Bliss *et al.,* 1986, McGahon & Lynch, 1996). It is possible that the inhibitory effect of IL-1β on LTP may be a consequence of its effects on release since it was also shown that IL-1β inhibited glutamate release *in vitro* (Murray & Lynch, 1998, Murray *et al.,* 1997). The underlying mechanism by which IL-1β elicits its inhibitory effects has been partially explored. One possibility is that the increase in the production of reactive oxygen species induced by IL-1β in the rat hippocampus (Lynch, 1998, O' Donnell *et al.*, 2000) inhibits glutamate release (Zoccarato *et al.,* 1995) but downstream cellular consequences of IL-1β binding to IL-1R1 is also likely to play a significant role.

IL-1β has shown to be involved in the activation of stress-activated protein kinases (SAPKs), p38 and c-jun N-terminal kinases (JNK). Activation of JNK and p38 are also associated with cellular responses to toxins and physical stresses in a

variety of cell types (Raingeaud *et al.*, 1995, Rizzo & Carlo-Stella, 1996, Uciechowski, *et al.*, 1996, Lu *et al.*, 1997). The aims of this study were threefold. Firstly, to assess the effects of a range of IL-1β concentrations on endogenous glutamate release in hippocampal syanptosomes *in vitro*. Secondly, to establish whether or not IL-1β induced an increase in the activity of the stress-activated protein kinases p38 and JNK in hippocampal synaptosomes, as previously shown in other cell types. The third objective was to assess whether or not the IL-1β-induced inhibition of glutamate release is associated with an increase in the activity of stress-activated protein kinases.

3.2 Methods:

3.2.1 Analysis of endogenous glutamate release *in vitro*:

Impure synaptosomal preparations (P_2) were prepared either from fresh hippocampus or dentate gyrus or frozen slices as previously described in section 2.3.2. The pellet was resuspended in oxygenated Krebs solution containing $CaCl_2$ (final concentration: 2mM). The synaptosomes were preincubated (1) for 20 min at 37°C with continuous oxygenation in the presence of IL-1β (final concentration: 10pg/ml, 100pg/ml 1ng/ml, 3.5ng/ml and 100ng/ml). (2) The ERK inhibitor PD 098059 (10μM). (3) The p38 inhibitor SB 203580 (100μm, 50μm), or (4) H_2O_2 (5mM). Samples were aliquotted onto filter paper in a filtration manifold and washed with Krebs solution containing $CaCl_2$ (2mM). The pretreated synaptosomes were incubated in the absence or presence of KCl (final concentration: 50mM). The filtrates were collected as described in section 2.6 and stored in -80°C for later analysis. An aliquot of the synaptosomal suspension was retained for analysis of protein concentration (see 2.4). The analysis of glutamate concentration in both standards (ranging from 12.5nM to 1μM) and samples is previously described in section 2.7. Glutamate concentration of samples was expressed as μmol glutamate/mg protein.

3.2.2 Activity of ERK, JNK and p38:

The activity of ERK, JNK and p38 in rat hippocampal and dentate gyri synaptosomes was assessed as described in detail in sections 2.8.1 and 2.8.2. Samples were prepared and loaded onto polyacrylamide gels to separate proteins which were transferred onto nitrocellulose paper and immunoblotted with anti-JNK, anti-p38, or anti-ERK antibodies, using the concentrations previously referred to in section 2.8.3. The antibody-protein complex was visualised using ECL detection, protein bands were quantified by densitometric analysis, and results were expressed as arbitrary units.

3.3 Results:

3.3.1 Effect of high concentrations of IL-1β on endogenous glutamate release in the *in vitro:*

Fig. 3.1 shows the effect of preincubation with varying high concentrations of IL-1β on glutamate release in rat hippocampal synaptosomes. 50mM KCl caused a significant increase in glutamate release in control (i.e. untreated) synaptosomes (*$p<0.05$; Student's t-test) from a mean value of 0.03 ± 0.0004 μmol/mg (SEM; n=6) to a mean value of 0.103 μmol /mg ± 0.02. Hippocampal synaptosomes preincubated with 1ng/ml IL-1β, showed a significant decrease in KCl-stimulated glutamate release compared with controls (+$p<0.05$; Student's t-test). Mean values were 0.038 ± 0.004 μmol/mg (SEM; n=6) and 0.039 ± 0.005 μmol/mg in unstimulated and KCl-stimulated conditions respectively. Similarly synaptosomes preincubated with 3.5ng/ml IL-1β, showed a significant decrease in glutamate release (+$p<0.05$; Student's t-test). Mean values were 0.034 ± 0.001 μmol/mg (SEM; n=6) and 0.032 ± 0.001 μmol/mg in unstimulated and KCl-stimulated conditions respectively. Synaptosomes treated with 100ng/ml IL-1β showed an increase in unstimulated release, where the mean values was 0.058 ± 0.009 μmol/mg (SEM; n=6) compared with 0.038 μmol /mg ± 0.0004 in the controls (*$p<0.05$; student's t-test). Although KCl-stimulated release was greater (0.088 ± 0.015 μmol/mg), this increase was not statistically different from unstimulated values.

3.3.2 Effect of PD 098059 on the IL-1β-induced inhibition of endogenous glutamate release *in vitro:*

The effect of preincubation with PD 098059 (final concentration: 10μM) and IL-1β (1ng/ml and 100ng/ml) was assessed on glutamate release in rat hippocampal synaptosomes. Fig. 3.2 indicates that KCl-stimulated release was significantly increased in control synaptosomes compared with unstimulated release (*$p<0.05$; Student's t-test). Mean values were 0.020 ± 0.005 μmol/mg (SEM; n=6) and 0.093 ± 0.03 μmol/mg for unstimulated and KCl-stimulated release respectively. Both concentrations of IL-1β (1ng/ml and 100ng/ml) caused a significant decrease in KCl-stimulated glutamate release compared with the control (+$p<0.05$; student's t-test). Synaptosomes preincubated with 1ng/ml IL-1β showed mean values obtained of 0.027 ± 0.005 μmol/mg (SEM: n=6) and 0.031 ±0.01 μmol/mg for unstimulated and KCl-stimulated release respectively. Preincubation with 100ng/ml IL-1β also significantly reduced KCl-stimulated release (+$p<0.05$; Student's t-test) to a mean value of 0.035 ± 0.01 μmol/mg (SEM: n=6) while the mean unstimulated release was

0.050 ± 0.01 μmol/mg. Addition of PD 098059 failed to block the inhibitory effect of IL-1β on glutamate release. Mean values were 0.050 ± 0.01 μmol/mg (SEM: n=6) and 0.035 ± 0.01 μmol/mg for unstimulated and KCl-stimulated release, respectively, in synaptosomes preincubated with both PD 098095 (10μm) and 1ng/ml IL-1β and 0.051 ± 0.01 μmol/mg (SEM: n=6) and 0.049 ± 0.009 μmol/mg for unstimulated and KCl-stimulated release respectively in synaptosomes preincubated with both PD 098059 (10μm) and 100ng/ml IL-1β

3.3.3 Effect of SB 203580 on the IL-1β-induced inhibition of endogenous glutamate release *in vitro:*

The effect of preincubation with SB203580 (final concentration: 100μM) and IL-1β (1ng/ml and 100ng/ml) on glutamate release in rat hippocampal synaptosomes was assessed. Fig. 3.3 indicates that KCl-stimulated release was significantly increased compared with unstimulated release in control synaptosomes (*$p<0.05$; Student's t-test). Mean values were 0.013 ± 0.00009 μmol/mg (SEM; n=6) and 0.029 ± 0.004 μmol/mg for unstimulated and KCl-stimulated release, respectively. Both concentrations of IL-1β (1ng/ml and 100ng/ml) caused a decrease in KCl-stimulated release compared with control. Synaptosomes preincubated with 1ng/ml IL-1β showed mean values of 0.019 ±0.003 μmol/mg (SEM: n=6) and 0.021 ± 0.004 μmol/mg for unstimulated and KCl-stimulated release, respectively. Synaptosomes preincubated with 100ng/ml IL-1β caused no increase in KCl-stimulated compared to controls. Mean values were 0.017 ± 0.002 μmol/mg (SEM: n=6) and 0.020 ± 0.003 μmol/mg for unstimulated and KCl-stimulated release respectively. Pretreatment with SB 203580 (100μm) did not affect the inhibition of release caused by 1ng/ml IL-1β; mean values were 0.021 ± 0.001 μmol/mg (SEM: n=6) and 0.015 ± 0.002 μmol/mg, respectively for unstimulated and KCl-stimulated release. Pretreatment with SB 203580 reversed the effect of 100ng/ml IL-1β and therefore there was a significant increase in KCl-stimulated release compared with unstimulated release from 0.014 ± 0.002 μmol/mg (SEM: n=6) to 0.024 ± 0.004 μmol/mg (*$p<0.05$; Student's t-test).

3.3.4 Effect of lower concentrations of IL-1β on endogenous glutamate release *in vitro*:

Fig. 3.4 shows that the effect of pretreating synaptosomes prepared from dentate gyrus with two concentrations of IL-1β (final concentrations: 10pg/ml and 100pg/ml). Endogenous glutamate release was significantly increased in control (i.e. untreated) synaptosomes (*$p<0.05$; Student's t-test). Mean values were 0.365 ± 0.044 μmol/mg (SEM; n=13) and 0.769 ± 0.166 μmol/mg for unstimulated and KCl-stimulated release, respectively. Synaptosomes preincubated with 10pg/ml IL-1β showed a slight increase in unstimulated release and significant decrease in KCl-stimulated release (+$p<0.05$; Student's t-test) and the mean values were 0.603 g ± 0.0943 μmol/mg (SEM; n=16) and 0.391 ± 0.054 μmol/mg for unstimulated and KCl-stimulated glutamate release respectively. Similarly, synaptosomes pretreated with 100pg/ml IL-1β caused a significant inhibitory effect on KCl-stimulated release (+$p<0.05$; student's t-test). The mean values were 0.498 ± 0.138 μmol/mg (SEM: n=7) and 0.318 ± 0.099 μmol/mg for unstimulated and KCl-stimulated glutamate release, respectively.

3.3.5 Effect of SB 203580 on IL-1β-induced inhibition of endogenous glutamate release *in vitro*:

The effect of SB 203580 (final concentration: 50μM) on the inhibitory effect of IL-1β (10pg/ml) on glutamate release in rat dentate gyrus was assessed. Fig. 3.5 indicates that KCl-stimulated release was significantly increased in control synaptosomes (*$p<0.05$; student's t-test. Mean values were 0.65 ± 0.07 μmol/mg (SEM; n=13) and 0.92 ± 0.09 μmol/mg for unstimulated and KCl-stimulated release release, respectively. Synaptospomes pretreated with IL-1β (10pg/ml) showed no increase in KCl-stimulated glutamate release compared to unstimulated release. The mean values were 0.87 ± 0.08 μmol/mg and 0.92 ± 0.13 μmol/mg for unstimulated and stimulated release, respectively. Synaptosomes preincubated with both SB 203580 (50μM) and IL-1β (10pg/ml) showed a significant increase in KCl-stimulated glutamate release where the mean values were 0.82 ± 0.01 μmol/mg and 1.01 ± 0.10 μmol/mg for unstimulated and KCl-stimulated release respectively (+$p<0.05$; student's t-test).

3.3.6 Phosphorylation of ERK in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

The phosphorylation of ERK in rat hippocampal synaptosomes was analysed by gel electrophoresis and immunoblotting. Fig 3.6 (B) shows that IL-1β (1ng/ml and 100ng/ml) caused no statistically significant change in ERK phosphorylation in hippocampal synaptosomes. One sample immunoblot is shown in fig. 3.6 (A). Mean values, in arbitrary units ,were 7149 ± 721 (SEM: n=7) for the control and 8051 ± 626 and 5873 ± 85 for ERK phosphorylation analysed in synaptosomes preincubated with 1ng/ml and 100ng/ml IL-1β, respectively.

3.3.7 Phosphorylation of JNK in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

The phosphorylation of JNK in rat hippocampal synaptosomes was analysed by gel electrophoresis and immunoblotting. Fig. 3.7 (A) shows one sample immunoblot that indicates that 1ng/ml IL-1β caused a slight decrease in JNK phosphorylation in hippocampal synaptosomes, whereas 100ng/ml IL-1β caused an increase. Densitometric analysis revealed that the effect of 1ng/ml IL-1β was not statistically significant, shown in Fig. 3.7 (B). The mean values expressed in arbitrary units were 26.55 ± 4.44 (SEM: n=6) and 15.88 ± 3.12 for control and 1ng/ml IL-1β preincubated synaptosomes, respectively. Synaptosomes preincubated with 100ng/ml IL-1β showed a significant increase in JNK phosphorylation for which the mean values were 37.69 ± 6.8 (*$p<0.05$; Student's t-test).

3.3.8 Phosphorylation of p38 in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

The phosphorylation of p38 in rat hippocampal synaptosomes was analysed by gel electrophoresis and immunoblotting. Fig. 3.8 (A) shows one sample immunoblot which indicates that 1ng/ml IL-1β had no effect on p38 phosphorylation in hippocampal synaptosomes, whereas 100ng/ml IL-1β caused an increase. Densitometric analysis revealed that 1ng/ml IL-1β did not exert a significant effect on p38 phosphorylation compared with the control (Fig. 3.8 (B). The mean values expressed in arbitrary units were 1473 ± 21 (SEM: n=5) and 1565 ± 19 for control and 1ng/ml IL-1β preincubated synaptosomes, respectively. Synaptosomes preincubated with 100ng/ml IL-1β showed a significant increase in p38 phosphorylation for which the mean value was 1958 ± 19.4 (*$p<0.05$; Student's t-test).

3.3.9 Phosphorylation of JNK in rat dentate gyrus synaptosomes: Effect of IL-1β *in vitro*

The phosphorylation of JNK in rat dentate gyrus synaptosomes was analysed by gel electrophoresis and immunoblotting. Fig. 3.9 (A) shows one sample immunoblot, which indicates that 10pg/ml IL-1β increased JNK phosphorylation in dentate gyrus synaptosomes, whereas 100pg/ml IL-1β had no effect. Densitometric analysis revealed that 10pg/ml IL-1β significantly increased JNK phosphorylation compared with the control (*$p<0.05$; Student's t-test; Fig. 3.9 (B)). The mean values, expressed in arbitrary units, were 32.2 ± 3.5 (SEM: n=7) and 40.5 ± 2.5 for control and 10pg/ml IL-1β preincubated synaptosomes respectively. Synaptosomes preincubated with 100pg/ml IL-1β showed no change in JNK phosphorylation compared with the control, for which the mean value was 33.7 ± 2.5.

3.3.10 Phosphorylation of p38 in rat dentate gyrus synaptosomes: Effect of IL-1β *in vitro*

The phosphorylation of p38 in rat dentate gyrus synaptosomes was analysed by gel electrophoresis and immunoblotting. Fig. 3.9 (A) shows one sample immunoblot which indicates that 10pg/ml IL-1β increased p38 phosphorylation in dentate gyrus synaptosomes, whereas 100pg/ml IL-1β had a similar effect. Densitometric analysis revealed that synaptosomes preincubated in the presence of 10pg/ml IL-1β showed significantly increased JNK phosphorylation compared with the control (*$p<0.05$; Student's t-test; Fig. 3.10 (B)). The mean values, expressed in arbitrary units, were 4460 ± 663 (SEM: n=4) and 5710 ± 732 for control and synaptosomes preincubated with 10pg/ml IL-1β, respectively. Synaptosomes preincubated with 100pg/ml IL-1β showed a similar, but not statistically significant, effect on p38 phosphorylation for which the mean value was 5420 ± 1089.

3.3.11 Effect of reactive oxygen species on endogenous glutamate release and stress-activated protein kinases *in vitro:* The use of H_2O_2 as an oxidant

Addition of 50mM KCl significantly increased glutamate release in control and untreated dentate gyrus synaptosomes (*$p<0.05$; Student's t-test). Fig. 3.11 (A) shows shows that mean values were 0.365 ± 0.043 μmol/mg (SEM; n=13) and 0.769

± 0.166 μmol/mg for unstimulated and KCl-stimulated glutamate release, respectively. Release of endogenous glutamate from H_2O_2-treated dentate gyrus synaptosomes (final concentration: 5mM) was significantly decreased compared with controls upon the addition of 50mM KCl to the reaction mixture (*$p<0.05$; Student's t-test). Mean values were 0.290 ± 0.088 μmol/mg (SEM; n=6) and 0.452 ± 0.092 μmol/mg for unstimulated and KCl-stimulated release respectively. The sample immunoblot shown in Fig. 3.11 (B), shows that H_2O_2 (5mM) increased JNK phosphorylation in dentate gyrus synaptosomes. Densitometric analysis revealed that JNK phosphorylation in synaptosomes preincubated with H_2O_2 was significantly increased (*$p<0.05$; Student's t-test). Mean values, expressed in arbitrary units, were 92.2 ± 17.0 (SEM; n=4) and 159.6 ± 21.3 for control and treated synaptosomes respectively. The sample immunoblot shown in fig.3.11 (C), shows that H_2O_2 (5mM) increased p38 phosphorylation in dentate gyrus synaptosomes. Densitometric analysis revealed that synaptosomes preincubated with H_2O_2 significantly increased (*$p<0.05$; Student's t-test) p38 phosphorylation. Mean values, expressed in arbitrary units, were 5093 ± 121 (SEM; n=6) and 8817 ± 977 for control and pretreated synaptosomes respectively.

Fig. 3.1 Effect of high concentrations of IL-1β on endogenous glutamate release *in vitro*:

Addition of 50mM KCl significantly increased glutamate release in control (i.e. untreated) hippocampal synaptosomes (*$p<0.05$; Student's t-test), glutamate release was significantly inhibited in synaptosomes which were pretreated with IL-1β (final concentration: 1ng/ml, 3.5ng/ml; *$p<0.05$, +$p<0.05$, Student's t-test). These results indicate a significant decrease in KCl-stimulated release in treated versus untreated synaptosomes. Preincubation in the presence of 100ng/ml IL-1β significantly increased unstimulated glutamate release (±$p<0.05$); student's t-test). Results are expressed as μmol glutamate/mg protein and are means ± SEM of 6 observations.

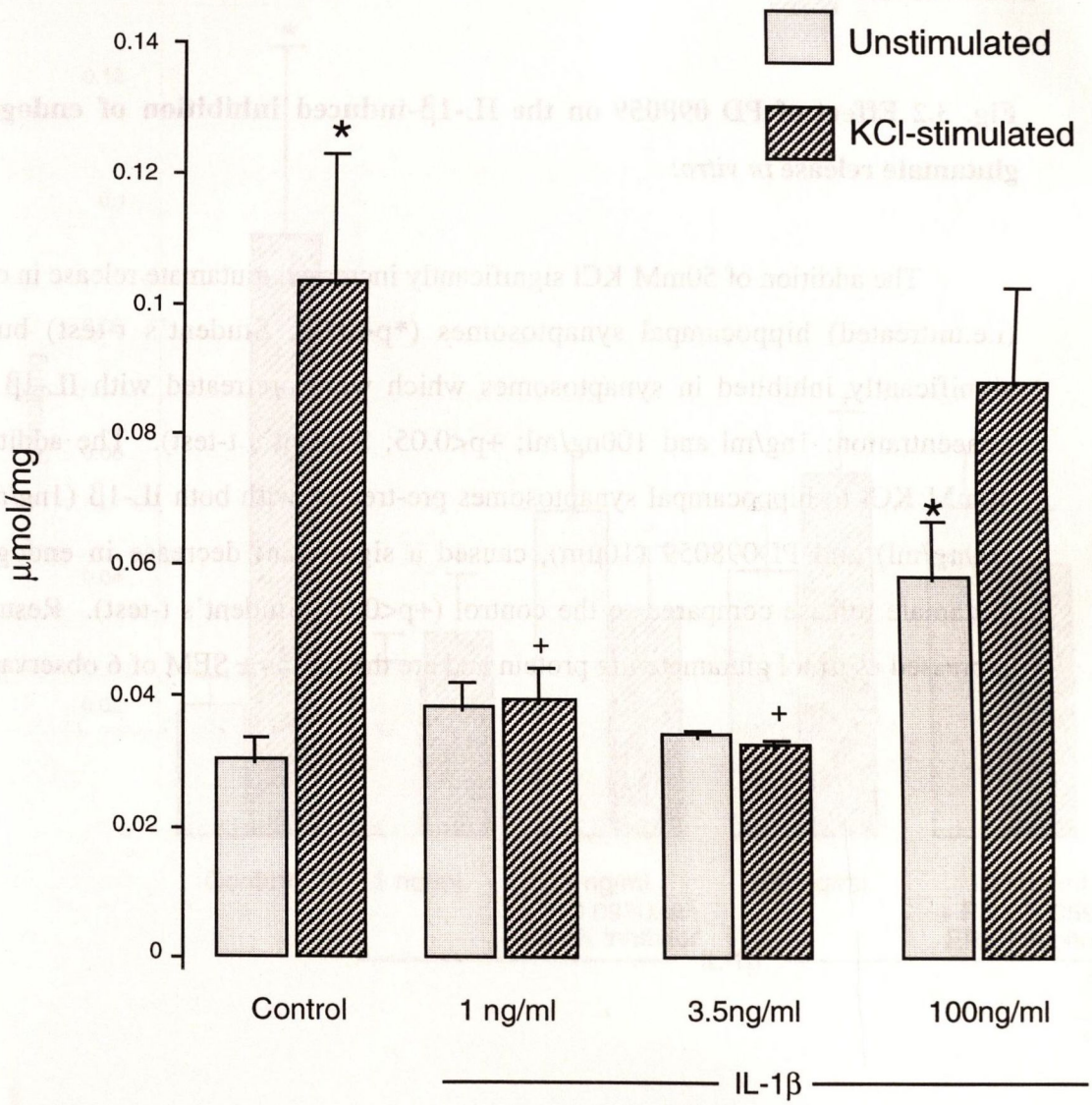

Unstimulated
KCl-stimulated
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
µmol/mg
*
+
+
*
Control
1 ng/ml
3.5ng/ml
100ng/ml
IL-1β

Fig. 3.2 Effect of PD 098059 on the IL-1β-induced inhibition of endogenous glutamate release *in vitro:*

The addition of 50mM KCl significantly increased glutamate release in control (i.e.untreated) hippocampal synaptosomes (*$p<0.05$; Student's t-test) but was significantly inhibited in synaptosomes which were pretreated with IL-1β (final concentration: 1ng/ml and 100ng/ml; +$p<0.05$; Student's t-test). The addition of 40mM KCl to hippocampal synaptosomes pre-treated with both IL-1β (1ng/ml and 100ng/ml) and PD098059 (10μm), caused a significant decrease in endogenous glutamate release compared to the control (+$p<0.05$; Student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 6 observations.

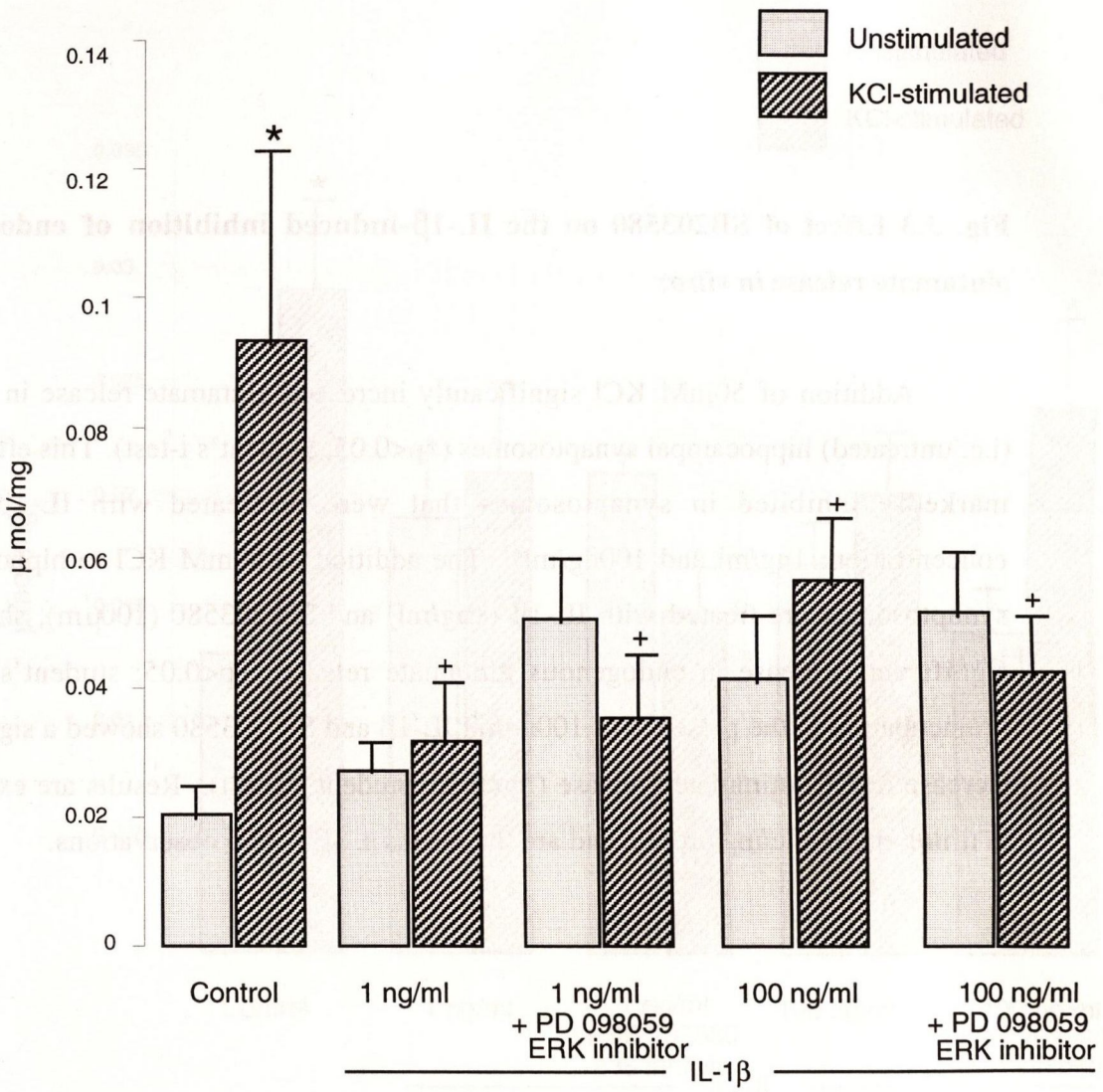

Unstimulated
KCl-stimulated
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
µ mol/mg
*
+
+
+
+
Control
1 ng/ml
1 ng/ml
+ PD 098059
ERK inhibitor
100 ng/ml
100 ng/ml
+ PD 098059
ERK inhibitor
IL-1β

Fig. 3.3 Effect of SB203580 on the IL-1β-induced inhibition of endogenous glutamate release *in vitro:*

Addition of 50mM KCl significantly increased glutamate release in control (i.e. untreated) hippocampal synaptosomes (*$p<0.05$; Student's t-test). This effect was markedly inhibited in synaptosomes that were pretreated with IL-1β (final concentration: 1ng/ml and 100ng/ml). The addition of 50mM KCl to hippocampal synaptosomes pre-treated with IL-1β (1ng/ml) and SB 203580 (100μm), showed a significant decrease in endogenous glutamate release (+$p<0.05$; student's t-test). Preincubation in the presence of 100ng/ml IL-1β and SB 203580 showed a significant increase in KCl-stimulated release (*$p<0.05$; student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 6 observations.

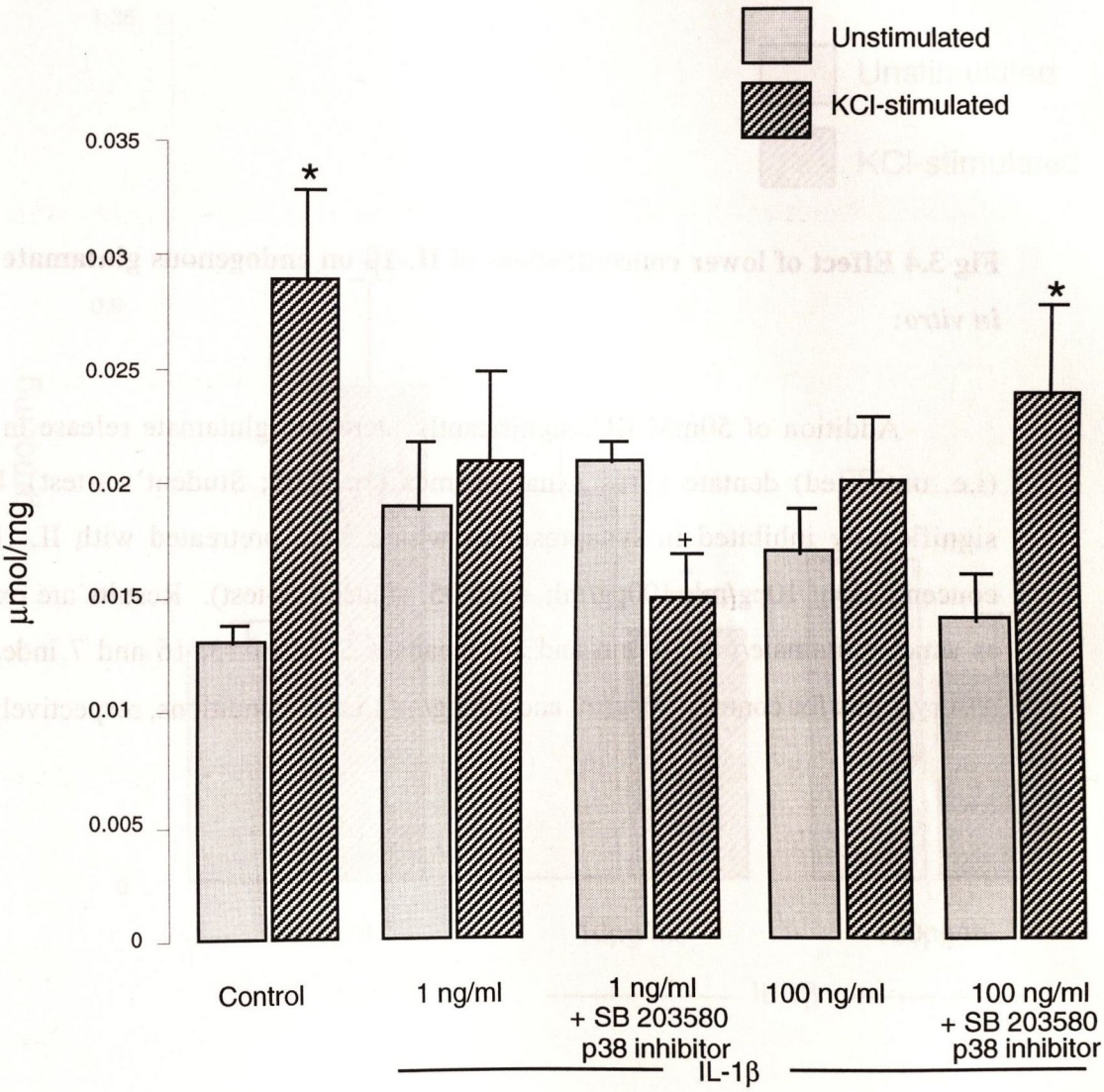

Unstimulated
KCl-stimulated
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
µmol/mg
*
*
+
Control
1 ng/ml
1 ng/ml
+ SB 203580
p38 inhibitor
100 ng/ml
100 ng/ml
+ SB 203580
p38 inhibitor
IL-1β

Fig 3.4 Effect of lower concentrations of IL-1β on endogenous glutamate release *in vitro*:

Addition of 50mM KCl significantly increased glutamate release in control (i.e. untreated) dentate gyrus synaptosomes (*$p<0.05$; Student's t-test), but was significantly inhibited in synaptosomes which were pretreated with IL-1β (final concentration: 10pg/ml, 100pg/ml; +$p<0.05$; student's t-test). Results are expressed as μmol glutamate/mg protein and are means ± SEM of 13, 16 and 7 independent observations for control, 10pg/ml and 100pg/ml treated conditions, respectively.

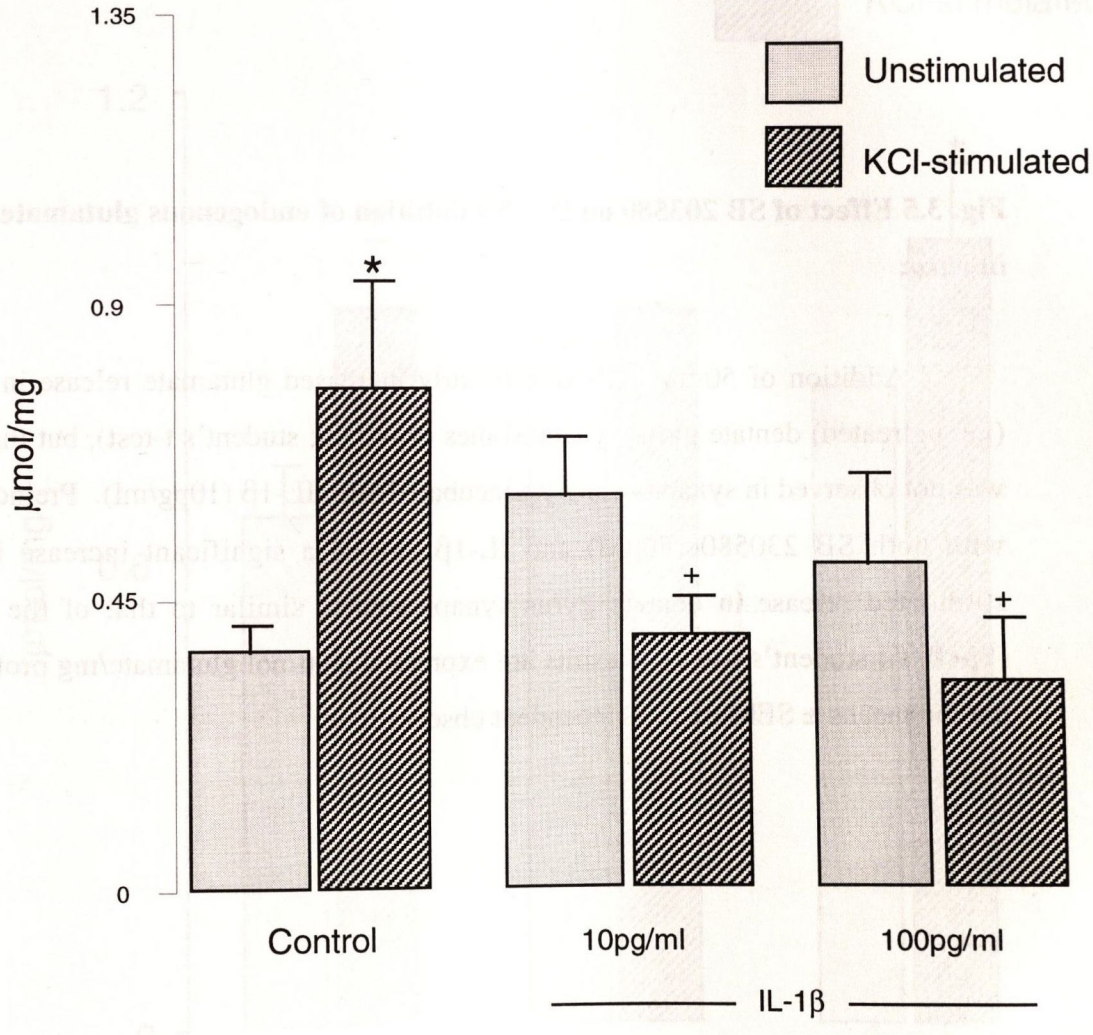

1.35
0.9
0.45
0
µmol/mg
Unstimulated
KCl-stimulated
*
+
+
Control
10pg/ml
100pg/ml
IL-1β

Fig. 3.5 Effect of SB 203580 on IL-1β-inhibition of endogenous glutamate release *in vitro*:

Addition of 50mM KCl significantly increased glutamate release in control (i.e. untreated) dentate gyrus synaptosomes (*$p<0.05$; student's t-test), but this effect was not observed in synaptosomes preincubated with IL-1β (10pg/ml). Preincubation with both SB 230580 (50μM) and IL-1β caused a significant increase in KCl-stimulated release in dentate gyrus synaptosomes, similar to that of the control (*$p<0.05$; student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 13 independent observations.

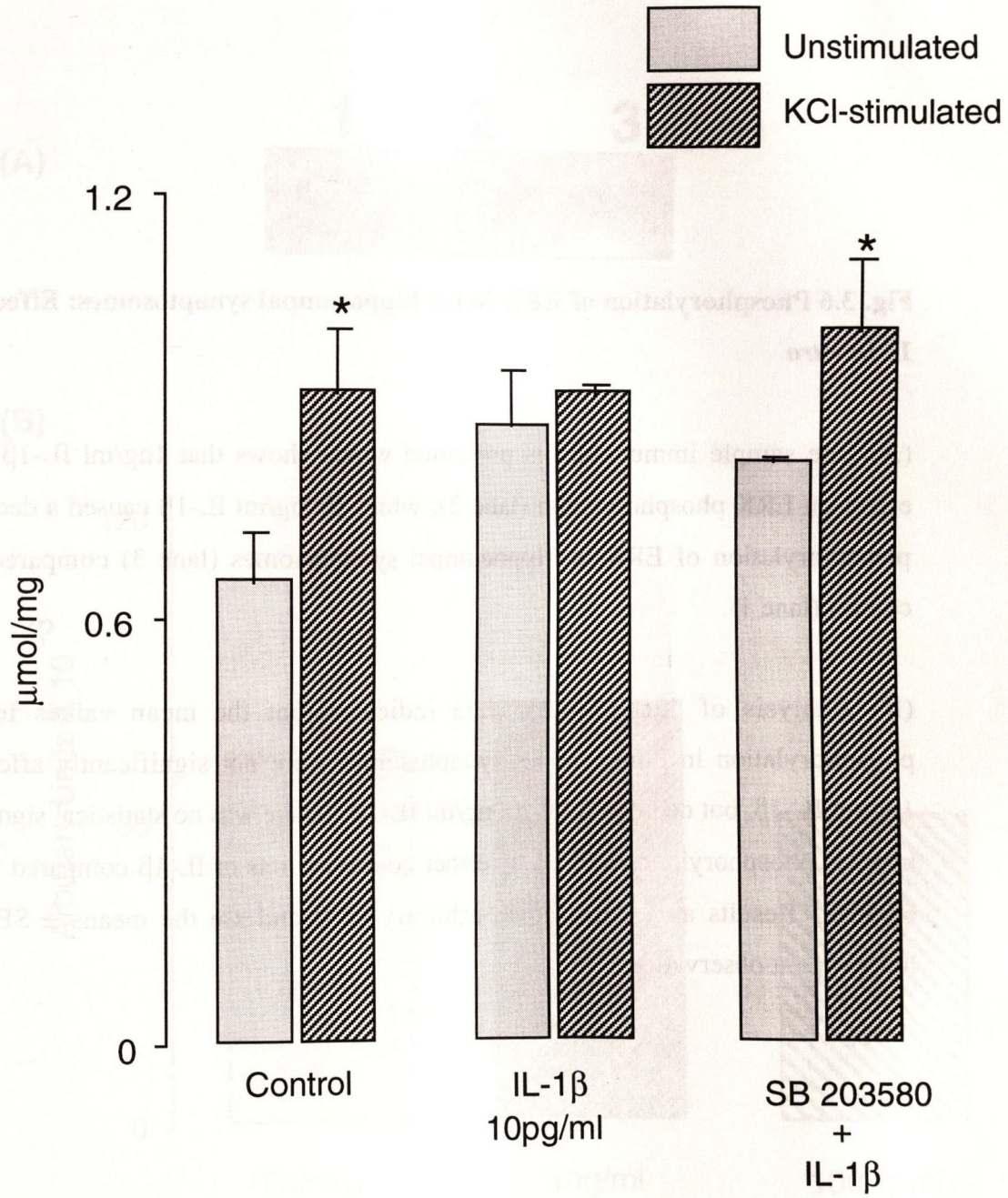
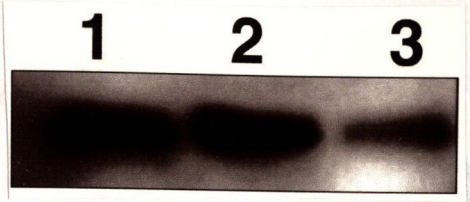

Unstimulated
KCl-stimulated
1.2
0.6
0
µmol/mg
*
*
Control
IL-1β
10pg/ml
SB 203580
+
IL-1β

Fig. 3.6 Phosphorylation of ERK in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

(A) One sample immunoblot is presented which shows that 1ng/ml IL-1β had no effect on ERK phosphorylation (lane 2), while 100ng/ml IL-1β caused a decrease in phosphorylation of ERK in hippocampal synaptosomes (lane 3) compared to the control (lane 1).

(B) Analysis of densitometric data indicates that the mean values for ERK phosphorylation in hippocampal synaptosomes were not significantly affected by 1ng/ml IL-1β, but decreased by 100ng/ml IL-1β. There was no statistical significance in ERK phosphorylation caused by either concentrations of IL-1β compared with the control. Results are expressed in arbitrary units and are the means ± SEM of 7 independent observations.

(A)


1
2
3

(B)

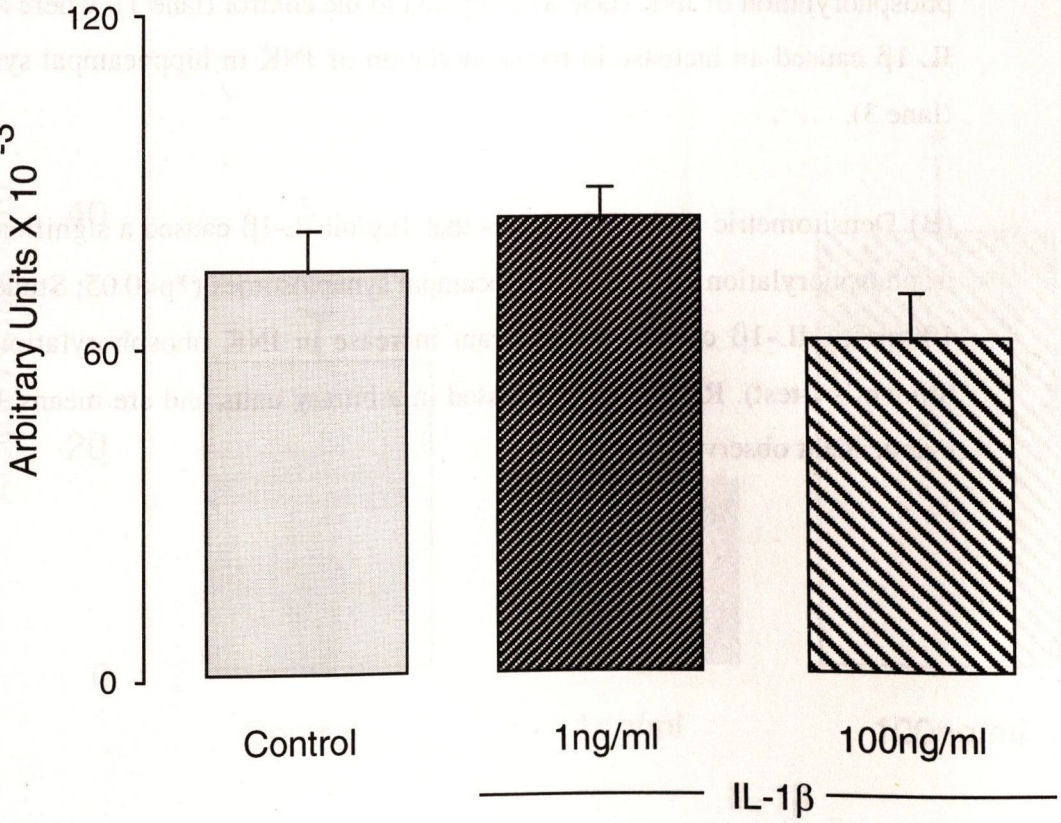

120
60
0
Arbitrary Units X 10 -3
Control
1ng/ml
100ng/ml
IL-1β

Fig. 3.7 Phosphorylation of JNK in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

(A) One sample immunoblot shows that 1ng/ml IL-1β caused a decrease in the phosphorylation of JNK (lane 2) compared to the control (lane 1), where as 100ng/ml IL-1β caused an increase in phosphorylation of JNK in hippocampal synaptosomes (lane 3).

(B) Densitometric analysis indicates that 1ng/ml IL-1β caused a significant decrease in phosphorylation of JNK in hippocampal synaptosomes (*$p<0.05$; Student's t-test). 100ng/ml IL-1β caused a significant increase in JNK phosphorylation (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are means ± SEM of 6 independent observations.

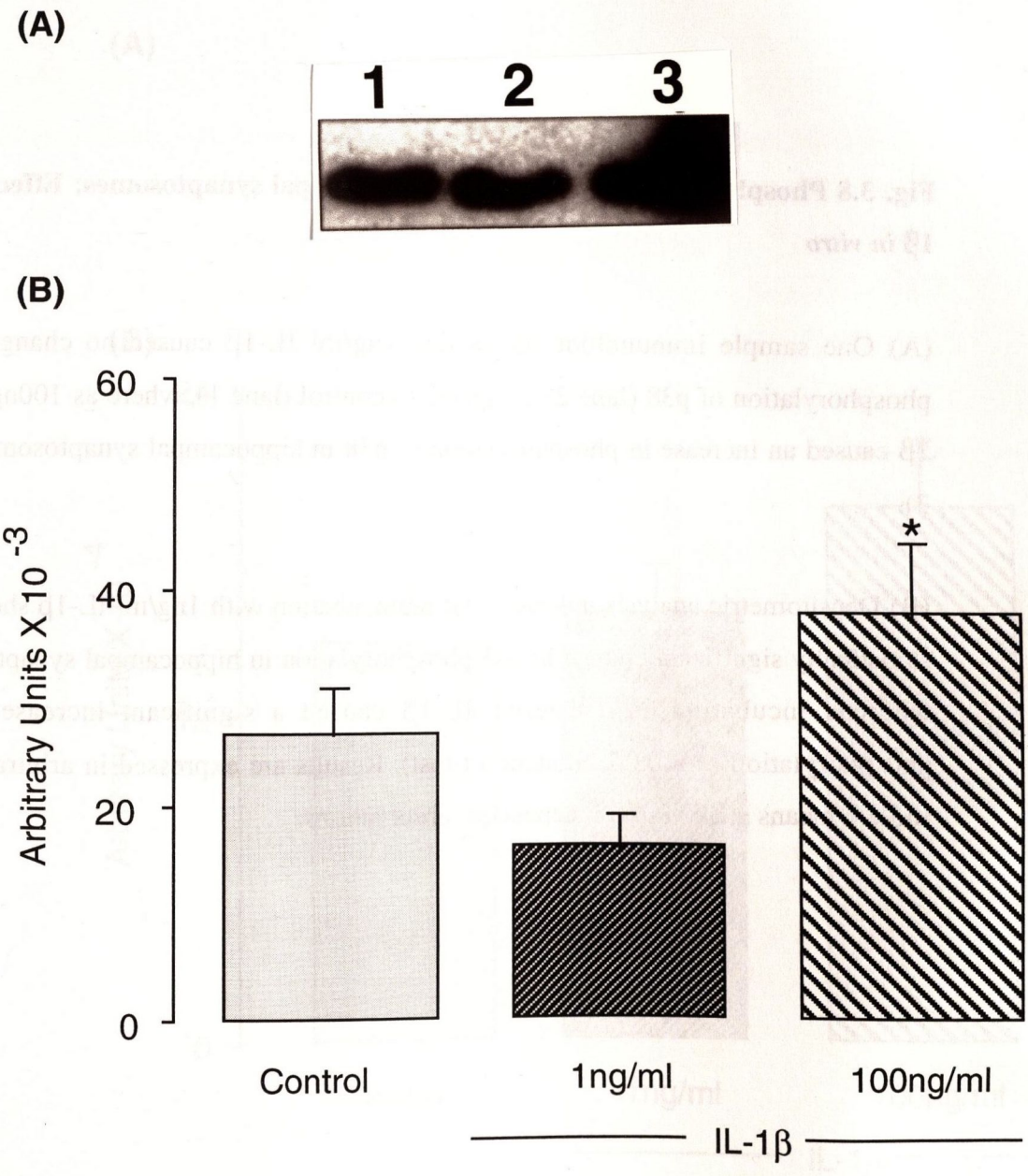

(A)
1
2
3
(B)
60
40
20
0
Arbitrary Units X 10 -3
*
Control
1ng/ml
100ng/ml
IL-1β

Fig. 3.8 Phosphorylation of p38 in rat hippocampal synaptosomes: Effect of IL-1β *in vitro*

(A) One sample immunoblot shows that 1ng/ml IL-1β caused no change in the phosphorylation of p38 (lane 2) compared to control (lane 1), where as 100ng/ml IL-1β caused an increase in phosphorylation of p38 in hippocampal synaptosomes (lane 3).

(B) Densitometric analysis indicates that preincubation with 1ng/ml IL-1β showed no statistically significant change in p38 phosphorylation in hippocampal synaptosomes, whereas incubation in 100ng/ml IL-1β caused a significant increase in p38 phosphorylation (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are means ± SEM of 5 independent observations.

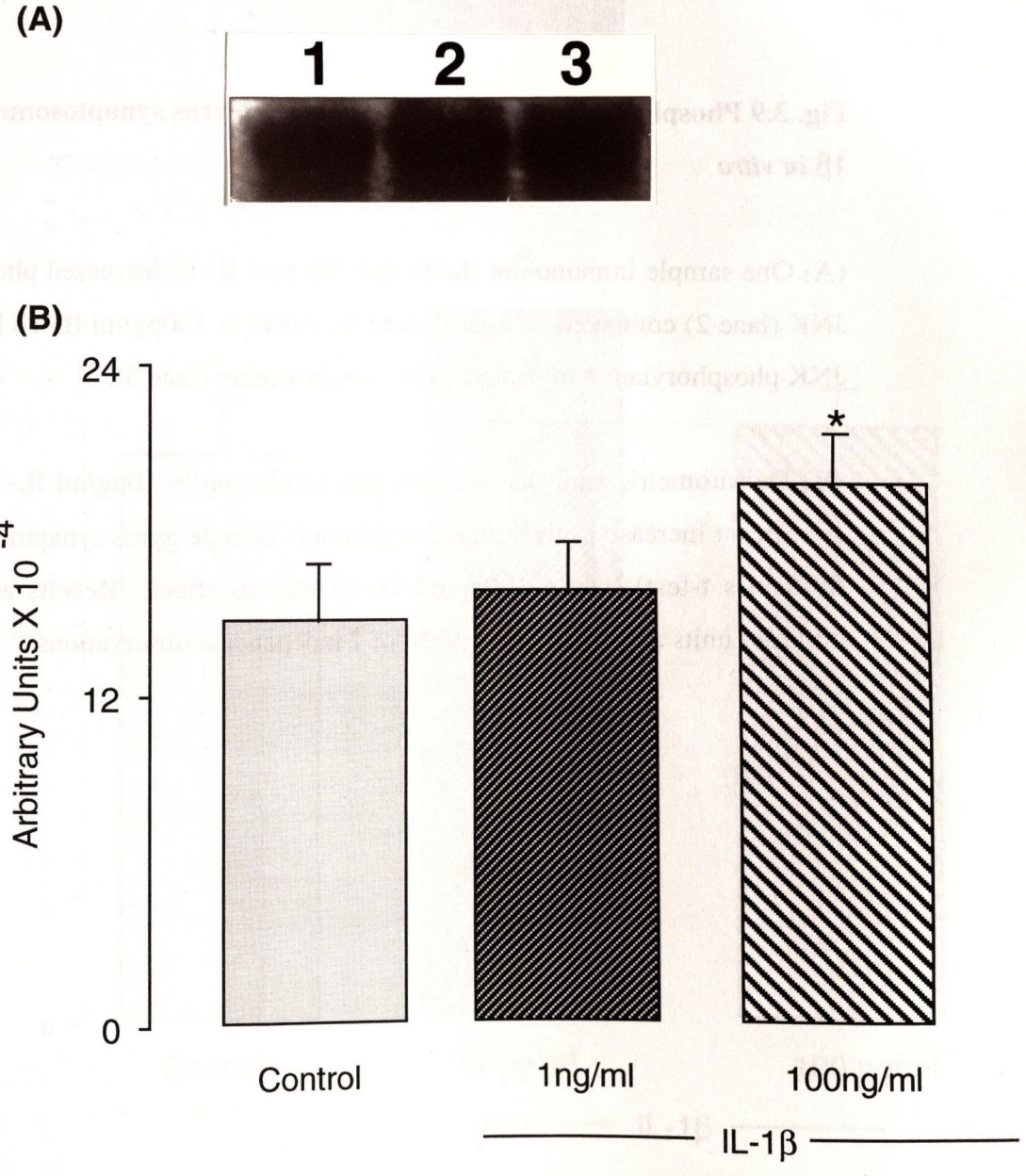
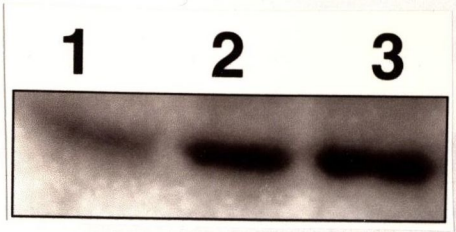

(A)
1
2
3
(B)
24
12
0
Arbitrary Units X 10 -4
*
Control
1ng/ml
100ng/ml
IL-1β

Fig. 3.9 Phosphorylation of JNK in rat dentate gyrus synaptosomes: Effect of IL-1β *in vitro*

(A) One sample immunoblot shows that 10pg/ml IL-1β increased phosphorylation of JNK (lane 2) compared to control (lane 1), where as 100pg/ml IL-1β had no effect on JNK phosphorylation in dentate gyrus synaptosomes (lane 3).

(B) Densitometric analysis indicates that incubation in 10pg/ml IL-1β resulted in a significant increase in JNK phosphorylation in dentate gyrus synaptosomes (*$p<0.05$; Student's t-test), while 100pg/ml IL-1β had no effect. Results are expressed in arbitrary units and are means ± SEM of 7 independent observations.

(A)



(B)

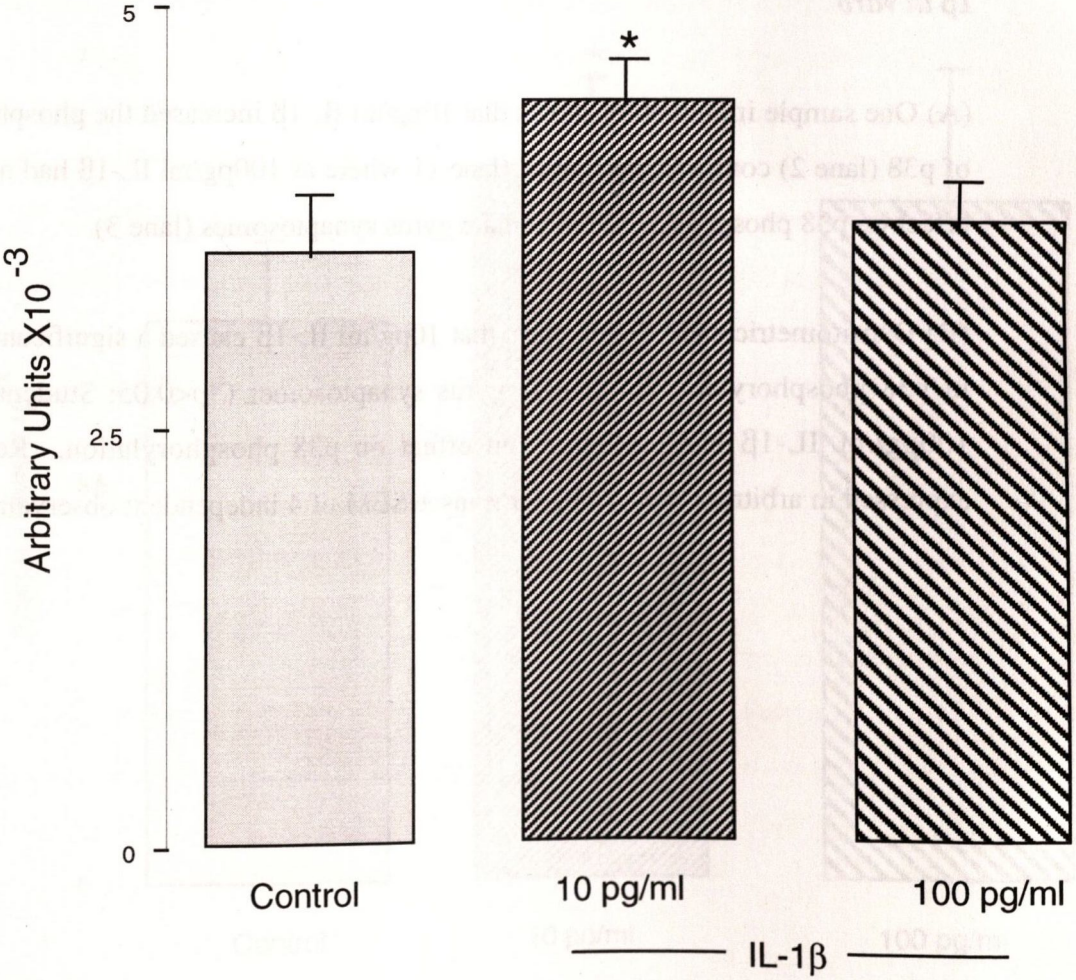


Fig. 3.10 Phosphorylation of p38 in rat dentate gyrus synaptosomes: Effect of IL-1β *in vitro*

(A) One sample immunoblot shows that 10pg/ml IL-1β increased the phosphorylation of p38 (lane 2) compared to control (lane 1), where as 100pg/ml IL-1β had no marked effect on p38 phosphorylation in dentate gyrus synaptosomes (lane 3).

(B) Densitometric analysis indicates that 10pg/ml IL-1β caused a significant increase in p38 phosphorylation in dentate gyrus synaptosomes (*$p<0.05$; Student's t-test). 100pg/ml IL-1β had no significant effect on p38 phosphorylation. Results are expressed in arbitrary units and are means ± SEM of 4 independent observations.

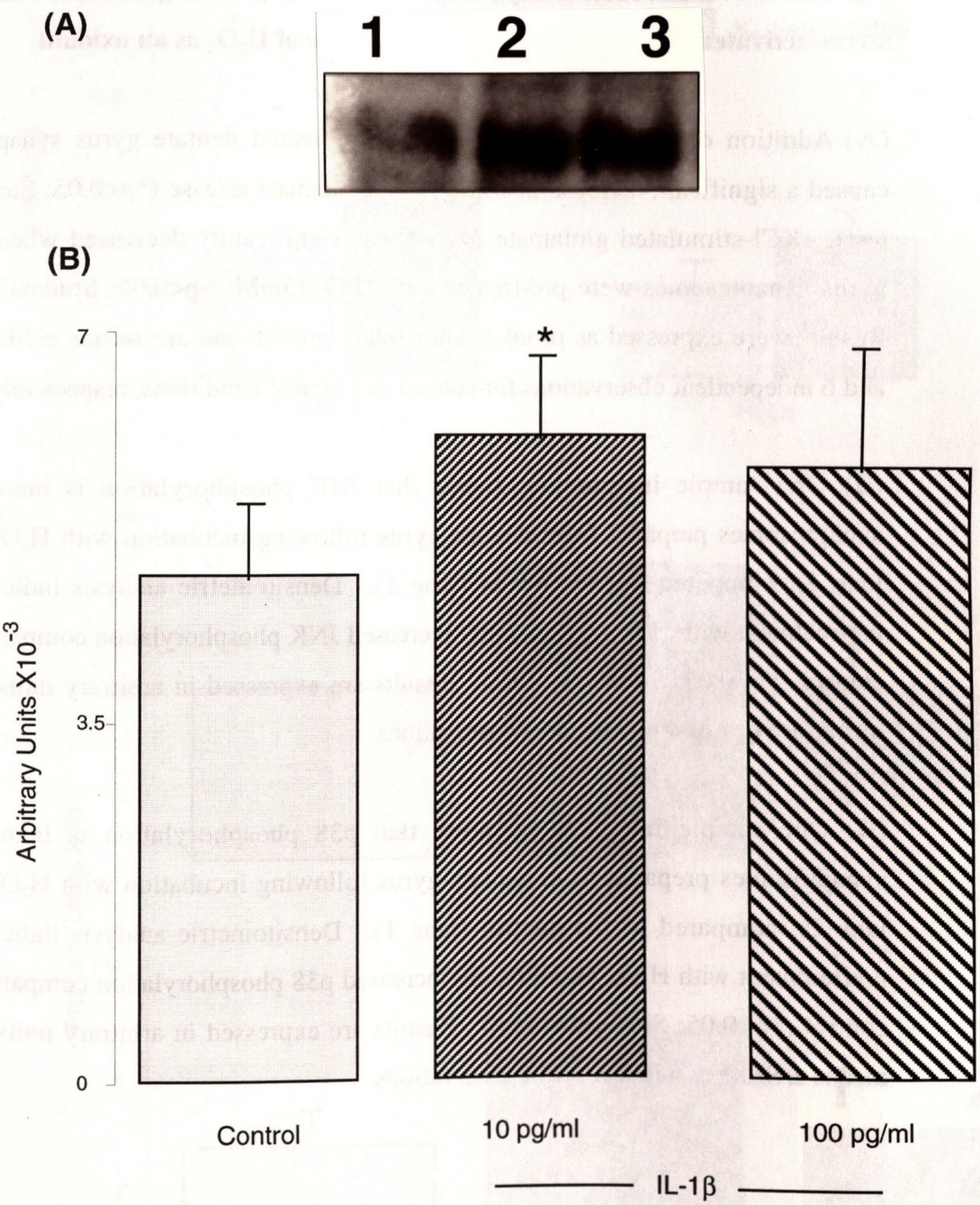
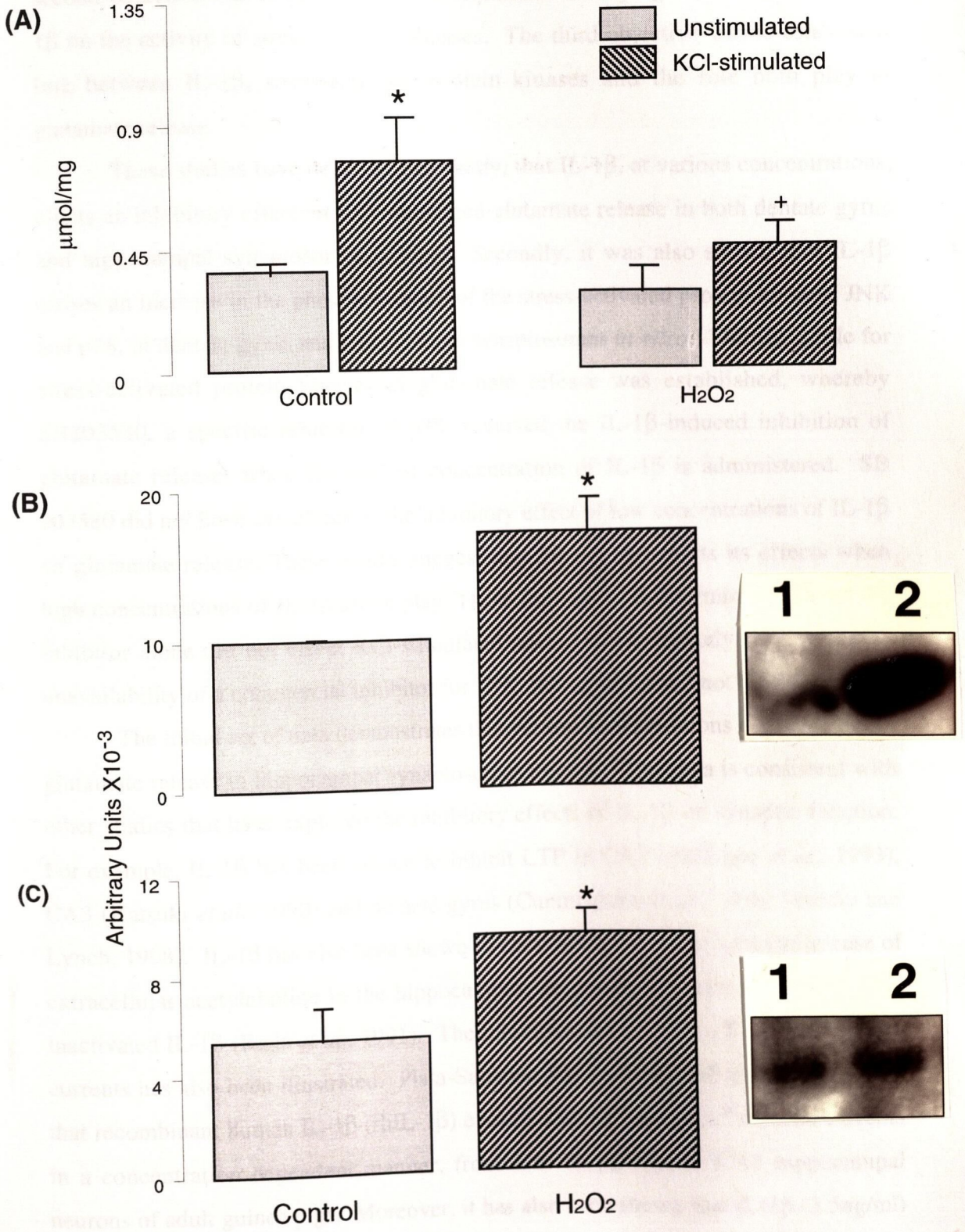

(A)
1
2
3
(B)
7
3.5
0
Arbitrary Units X10-3
*
Control
10 pg/ml
100 pg/ml
IL-1β

Fig. 3.11 Effect of reactive oxygen species on endogenous glutamate release and stress-activated protein kinases *in vitro*: The use of H_2O_2 as an oxidant

(A) Addition of 50mM KCl to control or untreated dentate gyrus synaptosomes caused a significant increase in endogenous glutamate release (*$p<0.05$; Student's t-test). KCl-stimulated glutamate release was significantly decreased when dentate gyrus synaptosomes were pre-treated with H_2O_2 (5mM; +$p<0.05$; Student's t-test). Results were expressed as μmol glutamate/mg protein and are means ± SEM of 13 and 6 independent observations for control and treated conditions, respectively.

(B) One sample immunoblot shows that JNK phosphorylation is increased in synaptosomes prepared from dentate gyrus following incubation with H_2O_2 (5mM; lane 2), compared to the control (lane 1). Densitometric analysis indicates that pretreatment with H_2O_2, significantly increased JNK phosphorylation compared to the control (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are means ± SEM of 4 independent observations.

(C) One sample immunoblot shows that p38 phosphorylation is increased in synaptosomes prepared from dentate gyrus following incubation with H_2O_2 (5mM; lane 2), compared to the control (lane 1). Densitometric analysis indicates that pretreatment with H_2O_2, significantly increased p38 phosphorylation compared to the control (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are means ± SEM of 6 independent observations.


(A)
1.35
0.9
0.45
0
µmol/mg
Unstimulated
KCl-stimulated
*
+
Control
H2O2
(B)
20
10
0
Arbitrary Units X10-3
*
1
2
(C)
12
8
4
0
*
1
2
Control
H2O2

3.4 Discussion:

The objectives of this study were threefold. The first objective was to investigate the effects of low and high concentrations of IL-1β on endogenous glutamate release in rat hippocampal and dentate gyrus synaptosomes *in vitro*. The second objective was to establish the consequences of varying concentrations of IL-1β on the activity of stress-activated kinases. The third objective was to establish a link between IL-1β, stress-activated protein kinases and the role both play in glutamate release.

These studies have demonstrated firstly, that IL-1β, at various concentrations, elicits an inhibitory effect on KCl-stimulated glutamate release in both dentate gyrus and hippocampal synaptosomes *in vitro*. Secondly, it was also shown that IL-1β causes an increase in the phosphorylation of the stress-activated protein kinases, JNK and p38, in dentate gyrus and hippocampal synaptosomes *in vitro*. Thirdly, a role for stress-activated protein kinases in glutamate release was established, whereby SB203580, a specific inhibitor of p38, reversed the IL-1β-induced inhibition of glutamate release, when the highest concentration of IL-1β is administered. SB 203580 did not have any effect on the inhibitory effect of low concentrations of IL-1β on glutamate release. These results suggest that SB 203580 exerts its effects when high concentrations of IL-1β are in play. These findings also determined that the p38 inhibitor alone did not effect KCl-stimulated release. Unfortunately because of the unavailability of a commercial inhibitor for JNK, this aspect could not be addressed.

The initial set of data demonstrates that various concentrations of IL-1β inhibit glutamate release in hippocampal synaptosomes *in vitro*. This data is consistent with other studies that have explored the inhibitory effects of IL-1β on synaptic function. For example, IL-1β has been shown to inhibit LTP in CA1 (Bellinger *et al.*, 1993), CA3 (Katsuki *et al.*, 1990) and dentate gyrus (Cunningham et al., 1996; Murray and Lynch, 1998). IL-1β has also been shown to exert inhibitory effects on the release of extracellular acetylcholine in the hippocampus, an effect that was not seen in heat inactivated IL-1β (Rada *et al.*, 1991). The inhibitory effect of IL-1β on Ca^{2+} channel currents has also been illustrated. Plata-Salamán and Ffrench-Mullen (1994) showed that recombinant human IL-1β (rhIL-1β) evoked a reduction in Ca^{2+} channel currents in a concentration dependent manner, from 2 to 30 pg/10μl in CA1 hippocampal neurons of adult guinea pigs. Moreover, it has also been shown that IL-1β (3.5ng/ml)

exerts an inhibitory effect on Ca^{2+} influx and KCl-stimulated release in rat hippocampal synaptosomes (Murray *et al.*, 1997). In addition, it has been showed that IL-1β (5ng/ml) reduced $^{45}Ca^{2+}$ influx into cortical synaptosomes and inhibited Ca^{2+} channel activity in cultured cortical neurons (MacManus *et al.*, 2000).

The method used to evaluate the concentration of endogenous glutamate in these studies was recently established (Kelly & Lynch, 1998, Ordronneau *et al.*, 1991) which replaced the method previously used to assess IL-1β-induced effects on glutamate release that involved prelabelling tissue with radioactive glutamate (Murray & Lynch, 1997). This method was revealed to be problematic as the equilibrium of [^{3}H]-glutamate within vesicular stores was low and background release of [^{3}H]-glutamate under basal conditions was high. Another drawback was that glutamate becomes rapidly metabolised to GABA and measurement of radioactivity revealed up to 20% labelling of GABA. These problems tended to skew data and render them less specific than required. Measurement of endogenous glutamate, which is carried out in the present experiments, overcomes these difficulties.

The data presented here show that concentration of glutamate measured varies between experiments to a certain extent. Among the factors that can be attributed to this is whether or not analysis of glutamate is carried out in either whole hippocampus or solely in dentate gyrus. The initial set of data assessed glutamate release in hipppocampal synaptosomes, followed by analysis in dentate gyrus synaptosomes. An additional variable is that concentrations of proteins varied between experiments; thus expression of concentration of glutamate per unit of protein introduced variability.

In the initial experiments, concentrations of IL-1β (1ng/ml, 3.5ng/ml and 100ng/ml) were quite high and were chosen on the basis of reported effects of IL-1β in the literature (Murray et al., 1997, Campbell & Lynch, 1998). For example, both *in vitro* and *in vivo* studies have used concentrations of IL-1β such as 3.5ng/ml, which caused inhibition of glutamate release and Ca^{2+} influx in hippocampal synaptosomes (Murray et al., 1997) and Ca^{2+} influx in cortical synaptosomes (Campbell & Lynch, 1998). However at the higher concentration of 100ng/ml, IL-1β was shown to enhance the KCl-induced rise in Ca^{2+} in cortical synaptosomes (Campbell & Lynch, 1998). Other reports reports that higher concentrations of IL-1β (7.5-50μg/kg) inhibit

Ca^{2+} channel currents in hippocampal neurons (Rada *et al.*, 1991). The results of this study with high concentrations of IL-1β broadly support these previous reports.

Glutamate release was assessed in the presence of lower concentrations of IL-1β (10pg/ml and 100pg/ml) which were also found to induce an inhibitory effect on KCl-stimulated release. These concentrations were used for the remainder of the study. The rationale behind this decision stems from various reports. Plata-Salaman and Ffrench-Mullen (1991) reported that at low concentrations, IL-1β (1.97, 7.9, and 31.2 pg/10μl) caused an inhibitory effect on Ca^{2+} channel currents in hippocampal neurons. In the same study it was also shown that higher concentrations did not further depress Ca^{2+} channel currents. It was hypothesised that IL-1 receptor numbers or intracellular messengers may be involved (Plata-Salaman & Ffrench-Mullen, 1991). IL-1 receptors in any cell type are low in number and the maximum biological activity appears only to involve 1-10% of the receptors (Dower *et al.*, 1992). The low concentrations of IL-1β (10 and 100pg/ml) used are within ranges observed in pathophysiological conditions such as Alzheimer's disease (Cacabelos *et al.*, 1991) and bacterial meningitis (Jacobs & Tabor, 1990).

The second aim of this study was to attempt to correlate the relationship between the IL-1β-induced inhibition of KCl-stimulated glutamate release and the stress-activated protein kinases p38 and JNK. Previous *in vitro* studies have shown that IL-1β stimulates the activity of JNK and p38 in various cell types. For example, IL-1β has been reported to increase activity of JNK in human glomerular mesangial (Uciechowski *et al.*, 1996) and HeLa (Raingeaud *et al.*, 1995) cells. IL-1β-induced activation of p38 has been reported in Chinese hamster CC139 (Guay *et al.*, 1997) and HeLa (Raingeaud *et al.*, 1995) cells. The data presented here is consistent with these findings since IL-1β, both at high and low concentrations, activate JNK and p38 in hippocampal synatosomes *in vitro*. The effect of IL-1β on ERK activation was also assessed. ERK is associated with cell growth and differentiation and is activated by stimuli which are different than those that activate p38 and JNK, such as mitogenic factors (Wang *et al.*, 1998, Jarvis *et al.*, 1996). It has previously been hypothesised that ERK activation increases glutamate release and plays a role in the induction of LTP (McGahon *et al.*, 1999). The present data indicates that IL-1β had no effect on ERK activity in hippocampal synaptosomes *in vitro* in contrast to the effect on the activities of JNK and p38.

The results described above demonstrate coupled inhibitory effects of IL-1β on endogenous KCl-stimulated glutamate release and stimulatory effects on the phosphorlyation of JNK and p38 in dentate gyrus and hippocampal synaptosomes *in vitro*. As a consequence of these findings, inhibitors of p38 and ERK were used to establish a link between protein kinase activation and glutamate release. SB 203580 is a specific inhibitor for p38 and PD098059 acts by inhibiting directly upstream from ERK. The findings revealed that PD 098059 (10μM) co-applied with IL-1β (1ng/ml and 100ng/ml) had no effect on the IL-1β-induced inhibition of glutamate, suggesting that ERK activity was not involved in the IL-1β-induced inhibition of glutamate release. The findings also indicated that SB 203580 (100μM), co-applied with IL-1β (100ng/ml) reversed the inhibitory effects of IL-1β on glutamate release in hippocampal synaptosomes. Furthermore, similar results showed that the incubation of dentate gyrus synaptosomes with SB 203580 (50μM) and IL-1β (10pg/ml) caused an increase in KCl-stimulated glutamate release, mirroring the data in the control experiment. In this study the effect of SB203580 alone was not assessed, but it has been previously reported by Coogan *et al* (1999b) that hippocampal slices pretreated with SB203580 did not lead to any significant changes in NMDA-receptor mediated transmission. This data suggests that the inhibitor alone has no effect on synaptic function.

Other studies investigating the role of p38 in synaptic plasticity have also used SB 203580. It has been shown that pretreatment of slices with SB230580 for one hour prior to IL-1β treatment, prevented the IL-1β-induced inhibition of both LTP and NMDA-receptor mediated field potentials in the rat dentate gyrus (Coogan *et al.*, 1999b). This result suggests that IL-1β inhibits LTP by activating p38. In contrast, English and Sweatt (1996, 1997) have established that ERK activation is necessary for the induction of LTP in CA1 hippocampal slices, while McGahon and co-workers (1999) have reported a role for ERK in LTP in the dentate gyrus. These results and the data presented in this study, suggest the involvement of the p38 MAPK cascade in modulating synaptic functions such as glutamate release. Unfortunately, the effect of JNK activity on glutamate release could not be investigated due to the lack of a commercially available inhibitor. For these reasons, the role JNK plays in synaptic functions has yet to be fully elucidated.

It has been suggested that the effects of IL-1β may be mediated through an increase in reactive oxygen species (ROS; Raingeaud *et al.,* 1995, Murray & Lynch, 1998b). The results presented in this study support this view, whereby H_2O_2, was used as an oxidant to assess the effect of ROS on endogenous glutamate release, JNK and p38 activity in dentate gyrus synaptosomes *in vitro*. The data here indicate that ROS inhibits KCl-stimulated release and evokes an increase in the phosphorylation of JNK and p38. H_2O_2, which leads to formation of the hydroxyl radical (Qin *et al.*, 1999) and therefore mimics the effect of IL-1β by stimulating JNK, p38 and inhibiting KCl-stimulated glutamate release *in vitro*. The results are consistent with reports that IL-1β increases ROS formation in hippocampal tissue (O' Donnell *et al.,* 2000). The effect of H_2O_2 on JNK, p38 and ERK on survival of HeLa cells has been assessed and it has been reported that the activity of the kinases determined the survival of the cells. ERK inhibition leads to an increase in apoptotic cells, whereas JNK and p38 inhibition enhanced cell survival (Wang *et al.,* 1997). However, other reports state that H_2O_2 strongly stimulates ERK and JNK (Tournier *et al.,* 1997). Although the mechanisms involved remains to be fully elucidated, it appears that stimulation of JNK activity may be through a pathway that involves arachidonic acid release (Tournier *et al.,* 1997). Other studies have shown that the activity of ERK is increased by arachidonic acid (AA) and the metabotropic glutamate receptor agonist, trans-1 amino-cyclopentyl-1,3-dicarboxylate (ACPD) *in vitro* . Both AA and ACPD increase glutamate release (McGahon *et al.,* 1996a) and the inhibition of ERK by PD 098059 has been shown to prevent the interaction between AA and ACPD on glutamate release (McGahon *et al.,* 1999). The data represented in this study suggest that IL-1β stimulate JNK and p38, perhaps through the formation of ROS. Consistent with this proposal, is the finding that stimulants of JNK and p38, such as UV radiation (Zhang *et al.,* 1997) and osmotic stress (Qin *et al.,* 1999) also induce formation of ROS in HeLa cells (Raingeaud *et al.*, 1995).

It has been previously documented that H_2O_2 has the ability to act as a messenger in the synaptic cleft (Edelman & Gally, 1992, Zoccarato *et al.,* 1995). The action of H_2O_2 on the exocytosis of KCl-stimulated glutamate release by cerebrocortical synaptosomes has been examined and it has been established that H_2O_2 induces a long-lasting inhibition of the exocytosis of glutamate by acting directly in the exocytotic process (Zoccarato *et al.,* 1995). The same group

established that H_2O_2 evokes an inhibitory effect on exocytotic glutamate release by promoting oxidation of specific thiol groups and modulating these events downstream of Ca^{2+} influx. Glutathione, an antioxidant, was shown to reverse the inhibitory effect of H_2O_2 on glutamate release, thus implicating this oxidant in synaptic inhibition (Zoccarato *et al.,* 1999).

The results obtained from this study demonstrate the IL-1β-induced inhibitory effects on endogenous glutamate release. One working model may be proposed as follows: pretreating synaptosomes with IL-1β at various concentrations may evoke an increase in ROS formation, thus changing the redox state of the pre-synaptic terminal. This change may be accompanied by an increase in the activation of stress-activated protein kinases, inhibiting KCl-stimulated glutamate release. The overall result suggests a role for stress-activated protein kinases in the IL-1β-induced inhibition of glutamate release

Chapter 4

The inhibitory effect of interleukin-1β on long-term potentiation is coupled with increased activity of stress-activated protein kinases.

4.1 Introduction

Long-term potentiation (LTP) in the perforant path-granule cell synapse is shown to be decreased in various experimental conditions such as ageing (McGahon *et al.*, 1997), behaviour (Murray & Lynch, 1998a) and following intracerebroventricularly (i.c.v.) injection of the proinflammatory cytokine, interleukin-1β (IL-1β; Bellinger *et al.*, 1993; Murray & Lynch, 1998a). One factor that is common to these experimental situations is an increase in the concentration of IL-1β in the hippocampus suggesting that such an increase might influence the expression of LTP. It has also been shown that IL-1β impairs the maintenance of LTP *in vitro*, in mossy fiber-CA3 pathway of the mouse hippocampus (Katsuki *et al.*, 1990), in the rat dentate gyrus (Cunningham *et al.*, 1996) and in the rat CA1 region of the hippocampus (Bellinger *et al.*, 1993).

It has previously been reported that the age-related impairment in LTP, coupled with an increase in the concentration of IL-1β, may be causally linked to an increase in reactive oxygen species (ROS) formation (Murray & Lynch, 1998a). Thus it has previously been reported that IL-1β increases the production of ROS in the rat hippocampus and hydrogen peroxide (H_2O_2), a potent oxidant, has been reported to induce an increase in IL-1β concentration (O' Donnell *et al.*, 2000), suggesting the involvement of a positive feed-back loop. ROS or free radicals are produced during normal metabolism, but in excess can result in tissue damage (Halliwell, 1992). Because the brain and nervous system are especially prone to oxidative stress, many studies have been undertaken in an attempt to better understand the significance of ROS production and how enhanced production can be overcome. *In vivo*, ROS are either sequestered by endogenous anti-oxidants (vitamin E, vitamin C or gluthathione) or metabolized by one of a number of enzymes (superoxide dismutase, gluthathione peroxidase or catalase). Thus a decrease in either enzyme or non-enzyme ant-oxidant strategies such as in ageing, might lead to increased accumulation of ROS, with the associated problems. One strategy used to manipulate ROS is diet. Diets enriched in polyunsaturated acids (McGahon *et al.*, 1999), dL-α-tocopheryl acetate (vitamin E) and ascorbic acid (vitamin C; O' Donnell & Lynch, 1998), docosahexanoic acid (Tuna Diet; O' Donnell *et al.*, 2000) have been shown to be protective against ROS. Rats fed on these variety of diets, overcome many problems that are associated with age, for example impairments in LTP, glutamate release and decreases in enzymatic and non-

enzymatic anti-oxidant levels are reversed (O' Donnell & Lynch, 1998; McGahon *et al.*, 1999; O' Donnell *et al.*, 2000). Other reports have shown that vitamin E has the ability to directly regulate H_2O_2 production in mitochondria (Chow *et al.*, 1999) and to reverse the age-related impairment in LTP (Murray & Lynch, 1998b)

A number of cellular events are triggered by IL-1β and ROS; in particular both have been shown to activate the stress-activated protein kinases, JNK and p38. Both kinases are stimulated by environmental stress including oxidative stress (Ip & Davis, 1995; Raingeaud *et al.*, 1995, Uciechowski *et al.*, 1996; Junger *et al.*, 1997), and activation leads to growth rest or even cell death (Park *et al.*, 1996; Maroney *et al.*, 1998). A considerable amount of evidence has indicated that both JNK and p38 are also stimulated by IL-1β (Derijard *et al.*, 1994; Raingeaud *et al.*, 1995; Rizzo-Carlo-stella, 1996; Uciechowski *et al.*, 1996; Lu *et al.*, 1997). Much of this evidence has been obtained from leukocytes and cultured cells, however there is a scarcity of evidence available for similar changes in neuronal tissue.

The aim of this series of experiments was to (1) examine the effect of IL-1β-injection on LTP and (2) assess the biochemical changes that may be associated with an increase in IL-1β. The effect of IL-1β-injection on the glutamate release and the activity of the stress-activated protein kinases, JNK and p38 was assessed in these circumstances. In addition, because of the known ability of IL-1β to generate ROS production, the effect of dietary supplementation on IL-1β-injection, on LTP, was also investigated. In this situation, endogenous glutamate, ROS formation, and the activity of JNK and p38 were examined.

4.2 Methods

4.2.1 Dietary supplementation

Young (2-4 months) male Wistar rats were randomly divided into groups (6 rats in each subgroup). They were housed 6 per cage. 2 groups were fed for 5 days with normal laboratory chow supplemented with a daily dose of dl-α-tocopheryl acetate (250mg/rat/day; 50% type SD Vitamin E, Beeline Healthcare, Dublin) dissolved in corn oil. Each gram of the dry powder contained 500mg of dl-α-tocopheryl acetate. Vitamin C (250mg/rat/day; Beeline Healthcare, Dublin) was added to the water supplied to the cages. The vitamin C product consisted of approximately 97% L-ascorbic acid. The other groups were fed for the same 5 day period on normal laboratory chow with added corn oil to ensure an isocaloric diet and nothing was added to the water offered to these rats. Daily food and water intake was monitored over a 5 day period before the start of the dietary supplementation. During the 5 day dietary phase animals were given 100% of their average daily food and water intake. Rats were weighed at the start and end of the experiment. General health and behaviour was monitored daily.

4.2.2 Intracerebroventricular (i.c.v.) injection

Rats were weighed and anaesthetized by an intraperitoneal (i.p.) injection of urethane (1.5g/kg). The head was positioned in a head holder within a stereotaxic frame. A midline incision was made with a scalpel and the skin was pulled back to reveal lamda and bregma. Interleukin-1β (IL-1β; 5µl; 3.5ng/ml) and saline (5µl; 0.9% saline) were administered in the manner described in section 2.5.4. Both IL-1β and saline were administered with a hamilton syringe into the ventricle. The effect of i.c.v. injection of IL-1β was examined in two groups, rats which were fed on the vitamin E/vitamin C diet and control diet, and rats fed only on normal laboratory chow.

4.2.3 Induction of LTP *in vivo*

Following i.c.v injection, a window of skull was removed to allow the insertion of the stimulating and recording electrodes in the layer of the dentate gyrus (see section 2.5.2. and 2.5.3). The depths of the electrodes were adjusted to obtain maximal responses in the cell body region. Electrophysiological recording commenced 30 min

after i.c.v. injection. Test shocks at a rate of 1/30 sec were delivered for 10 min prior to, and 40 min following tetanic stimulation (3 trains of stimuli; 250Hz for 200msec).

4.2.4 Tissue storage

At the end of the electrophysiological recording period, rats were killed as described (section 2.3). The hippocampus was removed and the tetanized and untetanized dentate gyri, as well as hippocampus proper, were dissected on ice and prepared for storage (section 2.3.1.).

4.2.5 Analysis of endogenous glutamate release *in vivo*

Impure synaptosomal preparations (P_2) were prepared from untetanized and tetanized dentate gyrus (section 2.3.2.). The pellet was resuspended in oxygenated Krebs solution containing $CaCl_2$ (final concentration: 2mM). Briefly, synaptosomes were aliquotted onto filter paper in a filtration manifold and washed with Krebs solution containing $CaCl_2$ (2mM), and incubated in the absence or presence of 50mM KCl. The filtrates were collected, stored, and analysized (section 2.6 and 2.7). Glutamate concentration of samples was expressed as μmol glutamate/mg protein.

4.2.6 Activity of JNK and p38

The activities of JNK and p38 were analysed in P_2 preparations obtained from hippocampal slices obtained from saline-pretreated and IL-1β-pretreated rats, some of which were fed on control and experimental diets. The analysis of JNK and p38 was assessed in rat dentate gyrus and hippocampal synaptosomes by the methods described in section 2.8.1 and 2.8.2. Briefly, proteins were separated by gel electrophoresis, transferred onto nitrocellulose paper and immunoblotted with antiphospho-JNK or anti-phospho-p38 antibodies (section 2.8.3.). The antibody-protein complex was visualised using ECL detection and protein bands were quantified by densitometric analysis and results were expressed as arbitrary units.

4.2.7 Determination of Reactive Oxygen Species (ROS) production

ROS formation was assessed in synaptosomes (see section 2.3.2) from hippocampal tissue obtained from saline- and IL-1β-treated rats fed on the control and vitamin-enriched diet. Ice-cold Tris buffer (1ml; 40mM pH 7.4) was added to

synaptosomal pellet in the presence of DCFH-DA and incubated for 15 min at 37°C. The dye-loaded synaptosomes were centrifuged at 13,000rpm at 4°C for 8 min. The pellet was resuspended in ice-cold Tris buffer (1.5ml; 40mM; pH 7.8). Fluorescence was measured at 525nm emission upon excitation at 488nm (for further details, see section 2.12). Results were expressed as μmol DCF/mg protein.

4.3 Results

4.3.1 Effect of IL-1β treatment on LTP *in vivo*

Analysis of the data indicates that i.c.v. injection of IL-1β (3.5ng/ml) inhibited LTP in perforant path-granule cell synapses (see fig. 4.1). The mean percentage increases in epsp slope in the 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min immediately prior to tetanic stimulation) were 158.6± 6.2 % (SEM; n=6) and 120.3± 3.1 % in the saline-pretreated and IL-1β-pretreated groups respectively. The mean percentage increases in epsp slope in the last 5 min of the experiment were 139.3 ± 1.6 % and 106.2 ± 1.3 % in the saline-pretreated and IL-1β-pretreated groups respectively.

4.3.2 Effect of IL-1β injection on endogenous glutamate release in dentate gyrus following the induction of LTP

Glutamate release was assessed in synaptosomes prepared from tetanized and untetanized dentate gyrus obtained from saline and IL-1β treated rats. Fig. 4.2 shows that the addition of 50mM KCl to synaptosomes prepared from untetanized dentate gyrus significantly increased glutamate release in saline-treated rats (*$p<0.05$; student's t-test for paired values). The mean values were 0.31 ± 0.09 μmol/mg (SEM: n=6) and 0.082 ± 0.10 μmol/mg for unstimulated and KCl-stimulated release respectively. A further enhancement of release was observed in synaptosomes prepared from tetanized tissue (**$p<0.001$; student's t-test for paired values). The mean values were 0.32 ± 0.10 μmol/mg and 1.6 ± 0.42 μmol/mg for unstimulated and KCl-stimulated release respectively. In contrast, KCl failed to stimulate release in synaptosomes prepared from untetanized dentate gyrus obtained from IL-1β-treated rats, where the mean values were 0.78 ± 0.21 μmol/mg (SEM: n=6) and 0.82 ± 0.20 μmol/mg for unstimulated and KCl-stimulated glutamate release, respectively. A similar result was seen in tetanized dentate gyrus from IL-1β-treated rats, whereby the

mean values were 0.74 ± 0.2 μmol/mg and 0.89 ± 0.25 μmol/mg for unstimulated and KCl-stimulated glutamate release respectively. The data also indicate that unstimulated glutamate release was significantly greater in synaptosomes prepared from both untetanized and tetanized (+$p<0.05$; student's t-test for paired values).

4.3.3 Effect of IL-1β injection on JNK activity following the induction of LTP

The activity of JNK was assessed in aliquots of synaptosomal tissue prepared from the dentate gyrus of rats that were pre-treated with IL-1β and saline, prior to the induction of LTP. The sample immunoblot shown in fig. 4.3 (A) shows that JNK activity in untetanized and tetanized tissue prepared from saline-treated rats was similar. The mean data obtained form densitometric analysis are shown in fig. 4.3 (B), where the mean values, expressed in arbitrary units, were 9.9 ± 2.9 (SEM: n=6) and 9.8 ± 1.06 for JNK activity in untetanized and tetanized tissue obtained from saline-treated rats, respectively. The sample immunoblot also indicates that the activity of JNK was markedly increased in untetanized tissue obtained from rats pre-treated with IL-1β. A slight increase was apparent in tetanized tissue, but this did not reach statistical significance. The mean values, obtained from densitometric analysis showed an IL-1β-induced increase in JNK activity (*$p<0.05$; student's t-test) such that the arbitrary units were 44.0 ± 0.7 (SEM: n=6) and 24.91 ± 2.73, for JNK activity assessed in untetanized and tetanized tissue obtained from IL-1β-pretreated rats, respectively.

4.3.4 Effect of IL-1β injection on p38 activity following the induction of LTP

Activity of p38 was assessed in aliquots of synaptosomal tissue prepared from the dentate gyrus of rats that were pre-treated with IL-1β and saline. The sample immunoblot shown in fig. 4.4 (A) shows that p38 activity in untetanized and tetanized tissue prepared from saline-treated rats was similar. The mean data obtained form densitometric analysis are shown in fig. 4.4 (B), where the mean values, expressed in arbitrary units, were 2.21 ± 0.37 (SEM: n=6) and 2.873 ± 0.32 for p38 activity in untetanized and tetanized tissue obtained from saline-treated rats, respectively. The sample immunoblot also indicates that the activity of p38 was markedly increased in untetanized and tetanized tissue obtained from rats pre-treated with IL-1β. This significant increase was also apparent in tetanized tissue. The mean values indicate

that p38 was significantly increased in untetanized (*p<0.05; student's t-test) and tetanized tissue (+p<0.05; studnet's t-test) obtained from IL-1β-pretreated rats, respectively. The mean values obtained from densitometric analysis and expressed in arbitrary units were 3.6 ± 0.2 (SEM: n=6) and 3.7 ± 0.5, for p38 activity assessed in untetanized and tetanized tissue obtained from IL-1β-pretreated rats, respectively.

4.3.5 Effect of IL-1β-treatment and dietary supplementation on reactive oxygen species

Fig. 4.5 indicates that there was an significant increase in ROS formation in hippocampal tissue obtained from IL-1β-treated rats fed on the control diet compared with saline-treated rats fed on the control diet (*p<0.05; student's t-test for paired values). The mean values were 14.3 ± 3.1 μmol DCF /mg protein (SEM; n=6), 37.0 ± 11.5 μmol DCF /mg protein and for saline- and IL-1β-treated rats fed on the control diet respectively. ROS formation was decreased in hippocampal tissue obtained from IL-1β-treated rats fed on the vitamin-enriched diet compared with IL-1β-treated rats fed on the control diet, but this difference did not reach statistical significance; the mean value was 29.6 ± 8.8 μmol DCF /mg protein (SEM; n=6). Similarly, hippocampal tissue obtained from saline-treated rats fed on the vitamin-enriched diet also showed a decrease in ROS formation. The mean value was 22.4 ± 5.4 μmol DCF /mg protein (SEM; n=6). These results suggest that the increase in ROS formation was attenuated by the vitamin-enriched diet. These data are presented with the permission of Dr. E. O' Donnell with whom I collaborated with on part of this study.

4.3.6 Effect of IL-1β treatment and dietary supplementation on LTP

Fig. 4.6 shows the effect of i.c.v. injection of IL-1β on LTP in rats fed on control diet and on a diet enriched in antioxidant vitamins E and C. The graph shows that delivery of a high frequency train of stimuli to the perforant path resulted in an immediate increase in the slope of the epsp in rats that were fed on the vitamin-enriched diet. Analysis of individual results reveals that all rats injected i.c.v. with saline sustained LTP for the duration of the experiment. The mean epsp slope was expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation. In rats fed on the control diet, the mean percentage changes in the population epsp slope (± SEM: n=6) in the 2 min immediately following tetanic

stimulation were 143.16 ± 5.46 % and 127.07 ± 2.01 % for saline-treated and IL-1β-treated rats respectively. The corresponding values in the last 5 min of the experiment were 120.1 ± 1.25 % and 107.48 ± 1.58 %. In rats fed on the vitamin-enriched diet, the mean percentage change in the mean population epsp slope (± SEM: n=6) in the 2 min immediately following tetanic stimulation were 140.96 ± 3.79 % and 119.25 ± 3.52 % for saline-treated and IL-1β-treated rats respectively. The corresponding values in the last 5 min of the experiment were 122.71 ± 9.65 % and 125.85 ± 2.38 %. Analysis of the data indicates that the maintenance of LTP was significantly decreased in the last 5 min of the experiment in IL-1β-treated rats fed on the control diet compared with the saline group fed on the vitamin-enriched diet ($p<0.01$; student's t-test).

4.3.7 The effect of IL-1β treatment and dietary supplementation on glutamate release

Glutamate release was assessed in synaptosomes prepared from tetanized and untetanized dentate gyrus obtained from rats which were fed on a control diet and vitamin-enriched diet and were IL-1β-pretreated and saline-pretreated prior to the induction of LTP. Fig. 4.7 shows that 50mM KCl significantly increase glutamate release in dentate gyrus synaptosomes prepared from saline-injected rats fed on the control diet from 0.185 ± 0.03 μmol/mg (SEM: n=6) to 0.27 ± 0.04 μmol/mg (*$p<0.05$; student's t-test). It was also seen that 50mM KCl significantly increased glutamate release in dentate gyrus synaptosomes obtained from rats fed on the vitamin-enriched diet from 0.166 ± 0.05 μmol/mg (SEM: n=5) to 0.22 ± 0.05 μmol/mg (*$p<0.05$; student's t-test). The KCl-induced enhancement of release was inhibited in tissue prepared from IL-1β-injected rats that were fed on the control diet. The mean values were 0.22 ± 0.02 μmol/mg (SEM: n=5) and 0.27 ± 0.03 μmol/mg for unstimulated and KCl-stimulated release. This inhibition was reversed in rats which were received the vitamin-enriched diet, whereby KCl-stimulated release was significantly greater than unstimulated release (*$p<0.05$; student's t-test) and the mean values were 0.23 ± 0.03 μmol/mg (SEM: n=6) and 0.36 ± 0.5 μmol/mg, respectively.

4.3.8 Effect of IL-1β treatment and dietary supplementation on JNK activity

Activity of JNK was assessed in aliquots of synaptosomal tissue prepared from the dentate gyrus of saline- and IL-1β-treated rats which were fed on the control and

vitamin-enriched diet. The sample immunoblot shown in fig. 4.8 (A) shows that JNK activity was increased in IL-1β-treated rats fed on the control diet. The mean data obtained from densitometric analysis are shown in fig. 4.8 (B). The mean values, expressed in arbitrary units, show that JNK activity was significantly increased from 13.53 ± 1.3 (SEM: n=6) to 31.12 ± 3.8 in saline-and IL-1β-treated rats fed on the control diet respectively (*$p<0.05$; student's t-test). There was no change in the activity of JNK in saline- and IL-1β-treated rats fed on the vitamin-enriched diet, where the mean values, obtained from densitometric analysis and expressed in arbitrary units, were 16.1 ± 2.9 (SEM: n=6) and 18.7± 2.9, respectively.

4.3.9 Effect of IL-1β treatment and dietary supplementation on p38 activity

Activity of p38 was assessed in aliquots of synaptosomal tissue prepared from the dentate gyrus of saline- and IL-1β-treated rats which were fed on the control and vitamin-enriched diet. The sample immunoblot shown in figure 4.9 (A) illustrate that p38 activity was increased in IL-1β-treated rats fed on the control diet. The mean data obtained from densitometric analysis are shown in fig. 4.9 (B). The mean values, expressed in arbitrary units, indicate that p38 activity was significantly increased from 9.4 ± 0.4 (SEM: n=6) to 12.1 ± 1.1 in saline-and IL-1β-treated rats fed on the control diet respectively (*$p<0.05$; student's t-test). There no increase in the activity of p38 in saline- and IL-1β-treated rats fed on the vitamin-enriched diet, where the mean values, obtained from densitometric analysis and expressed in arbitrary units, were 10.3 ± 1.2 (SEM: n=6) and 10.2 ± 1.5, respectively.

Fig. 4.1 Effect of IL-1β treatment on LTP *in vivo:*

Rats were anaesthetized by intraperitoneal injection of urethane (1.5g/kg). The rats were injected intracereboventricularly with either IL-1β (5μl; 3.5ng/ml) or saline (5μl; 0.9%). Test shocks were given at a rate of 1/30 sec, delivered for 10 min prior to tetanic stimulation and then LTP was induced by delivery of 3 high-frequency trains of stimuli. Stimulation at test shock frequency resumed and recordings continued for 45 min. Tetanic stimulation induced an immediate increase in the epsp slope in both saline-injected and IL-1β-injected rats, though this was attenuated following IL-1β injection. The mean slope decreased with time following IL-1β injection, so that the value was close to baseline at the end of the 45 min recording period. Sample recordings in the 5 min immmediately before tetanic stimulation are superimposed for saline-injected (left hand records) and IL-1β-injected (left hand records) rats. The scale bars represent 1 mV and 1 ms, SEM values are included for every tenth response. Mean epsp slope for each group is expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation and the data is presented as the mean change with time. Values on the 5 min pre-tetanus were normalized to 100%.

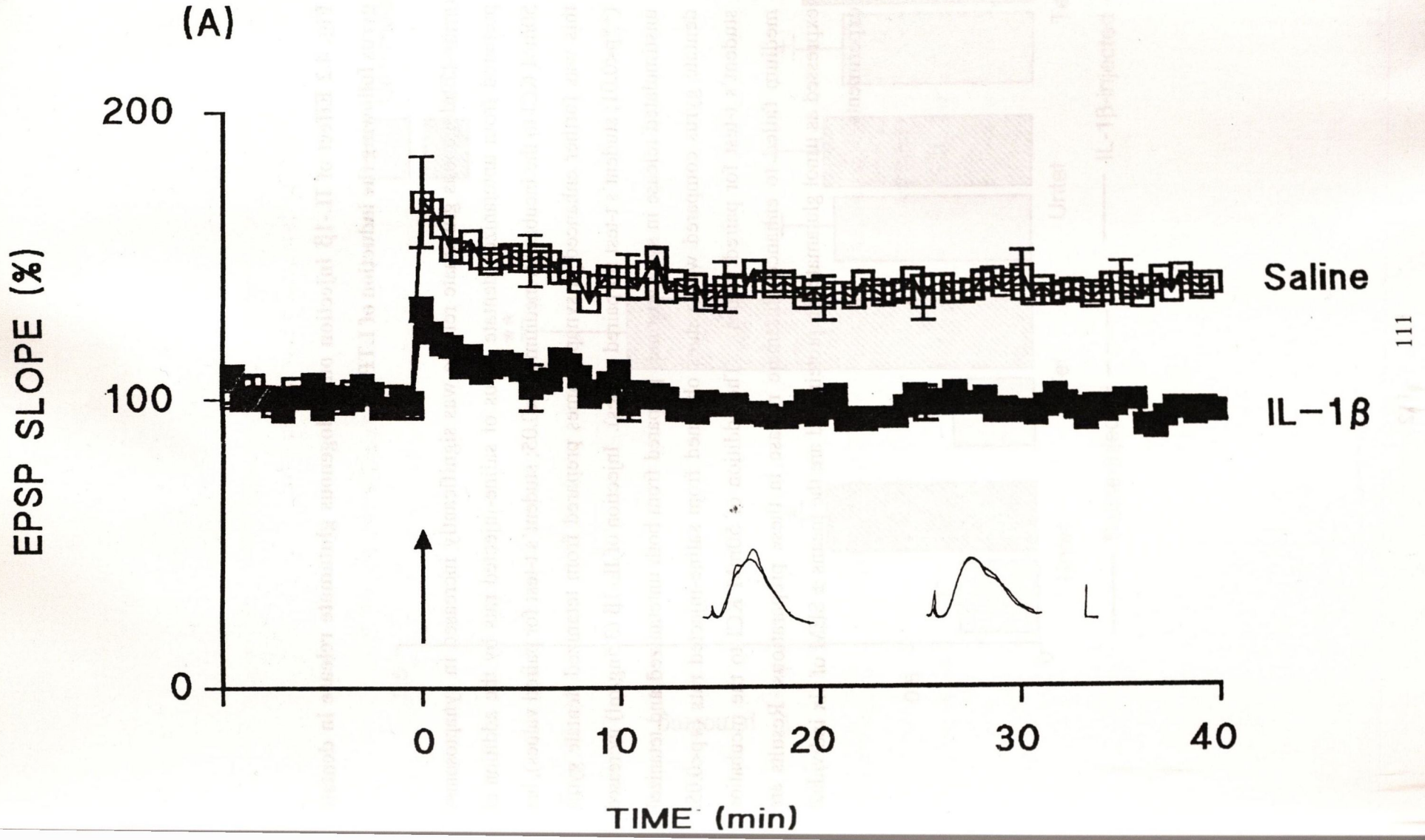

(A)
200
100
0
EPSP SLOPE (%)
Saline
IL-1β
L
0
10
20
30
40
TIME (min)

Fig. 4.2 Effect of IL-1β injection on endogenous glutamate release in dentate gyrus following the induction of LTP:

Endogenous glutamate release was significantly increased in synaptosomes prepared from untetanized dentate gyrus of saline-injected rats by the addition of 50mM KCl to the incubation medium (*$p<0.05$; student's t-test for paired values), but this was further enhanced in synaptosomes prepared from tetanized dentate gyrus (**$p<0.01$; student's t-test for paired values). Injection of IL-1β (3.5ng/ml) increased unstimulated release in synaptosomes prepared from both untetanized and tetanized dentate gyrus compared with values obtained from saline-injected rats (+$p<0.05$; student's t-test for paired values). The addition of 50mM KCl to the incubation medium failed to enhance glutamate release in these preparations. Results are expressed as μmol glutamate/mg protein and are the means ± SEM of six individual experiments.

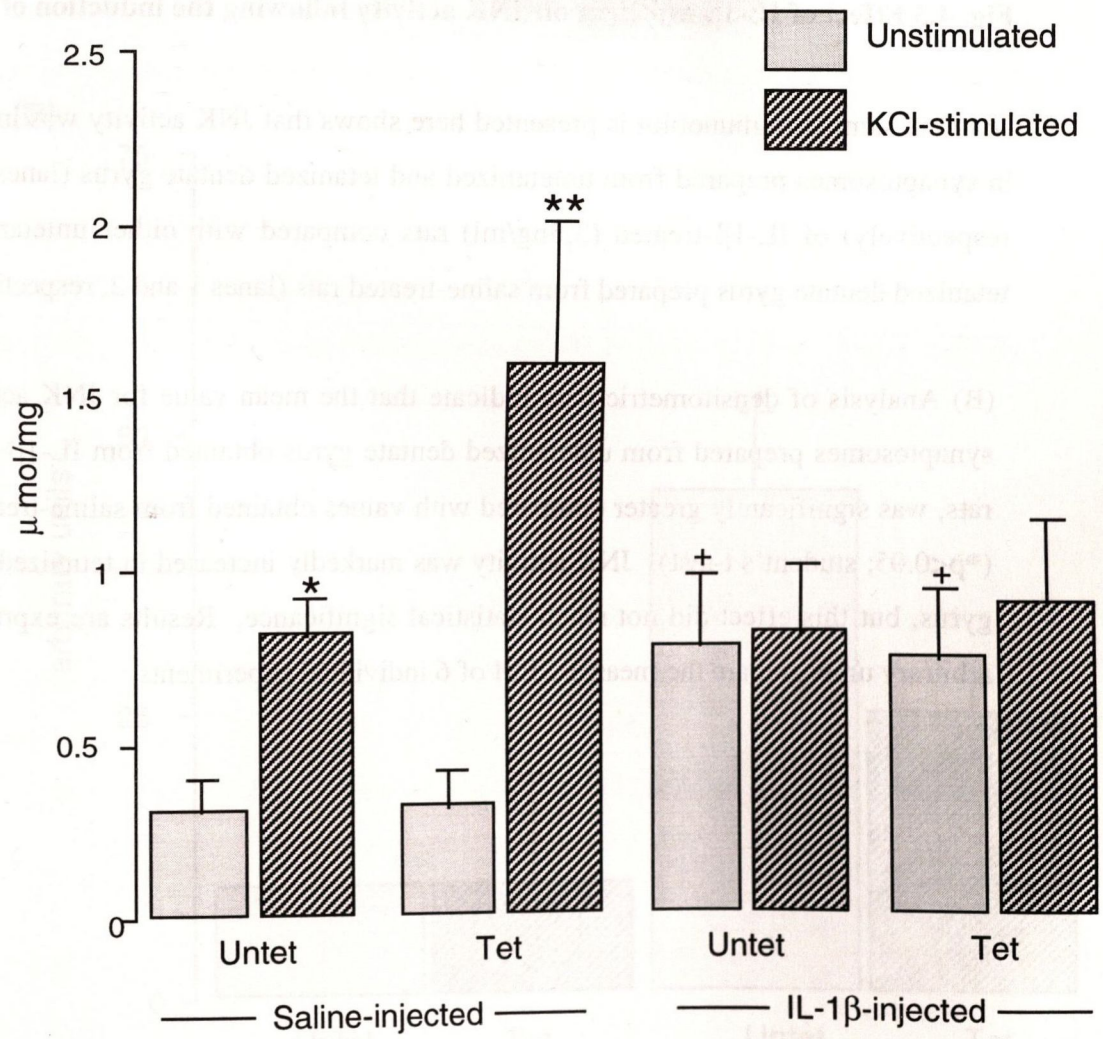

Unstimulated
KCl-stimulated
2.5
2
1.5
1
0.5
0
µ mol/mg
*
**
+
+
Untet
Tet
Untet
Tet
Saline-injected
IL-1β-injected

Fig. 4.3 Effect of IL-1β injection on JNK activity following the induction of LTP:

(A) One sample immunoblot is presented here shows that JNK activity was increased in synaptosomes prepared from untetanized and tetanized dentate gyrus (lanes 3 & 4, respectively) of IL-1β-treated (3.5ng/ml) rats compared with either untetanized or tetanized dentate gyrus prepared from saline-treated rats (lanes 1 and 2, respectively).

(B) Analysis of densitometric data indicate that the mean value for JNK activity in synaptosomes prepared from untetanized dentate gyrus obtained from IL-1β-injected rats, was significantly greater compared with values obtained from saline-treated rats (*$p<0.05$; student's t-test). JNK activity was markedly increased in tetanized dentate gyrus, but this effect did not reach statistical significance. Results are expressed as arbitrary units and are the mean ± SEM of 6 individual experiments.

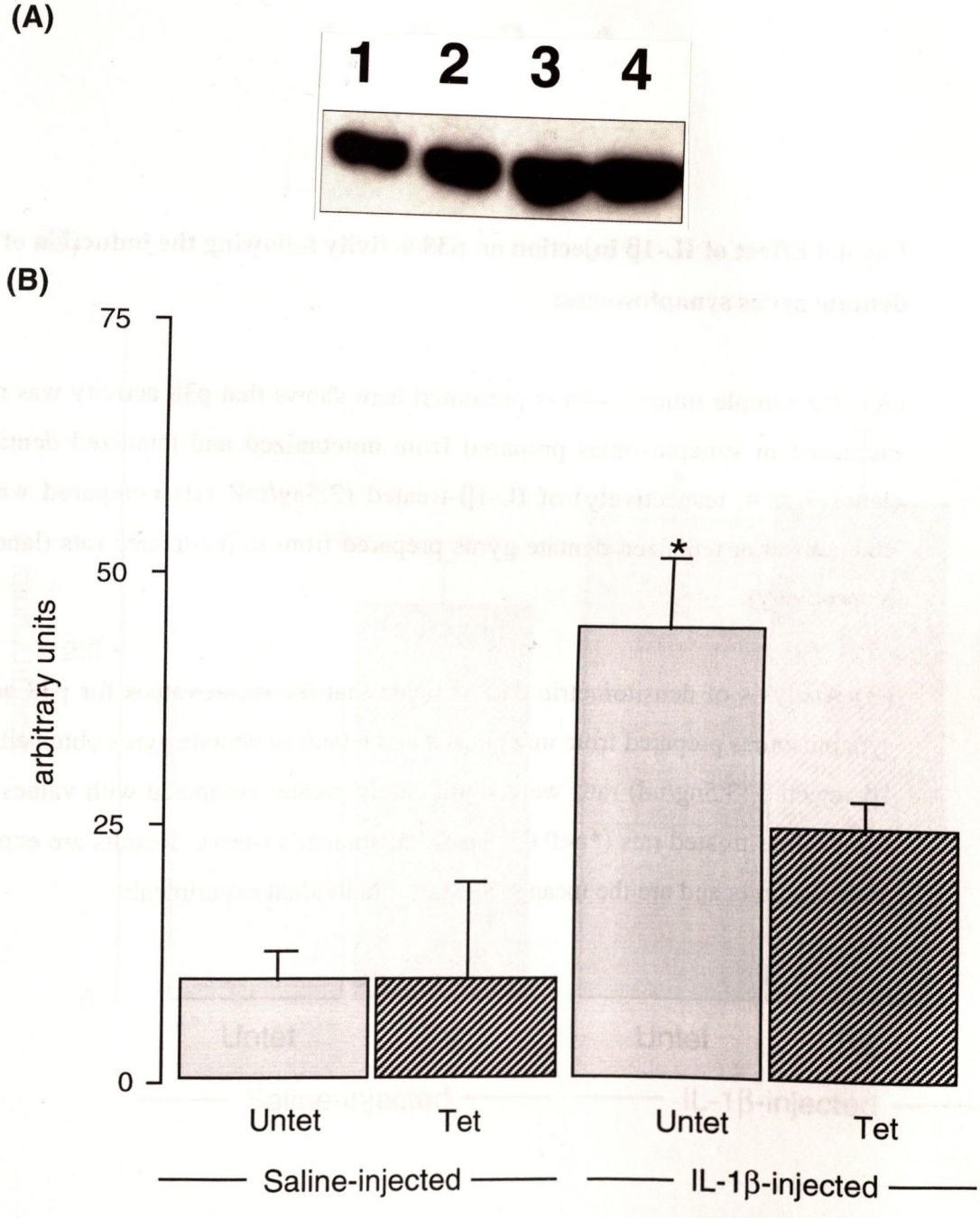
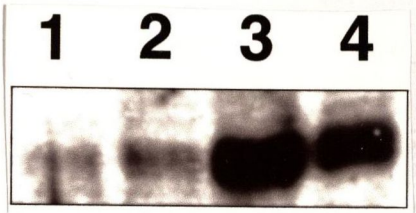

(A)
1 2 3 4
(B)
75
50
25
0
arbitrary units
*
Untet
Tet
Untet
Tet
Saline-injected
IL-1β-injected

Fig. 4.4 Effect of IL-1β injection on p38 activity following the induction of LTP, in dentate gyrus synaptosomes:

(A) One sample immunoblot is presented here shows that p38 activity was markedly increased in synaptosomes prepared from untetanized and tetanized dentate gyrus (lanes 3 & 4, respectively) of IL-1β-treated (3.5ng/ml) rats compared with either untetanized or tetanized dentate gyrus prepared from saline-treated rats (lanes 1 & 2, respectively).

(B) Analysis of densitometric data indicate that the mean values for p38 activity in synaptosomes prepared from untetanized and tetanized dentate gyrus obtained from IL-1β-injected (3.5ng/ml) rats, were significantly greater compared with values obtained from saline-treated rats (*$p<0.05$, +$p<0.05$; student's t-test). Results are expressed as arbitrary units and are the mean ± SEM of 6 individual experiments.

(A)


1
2
3
4

(B)

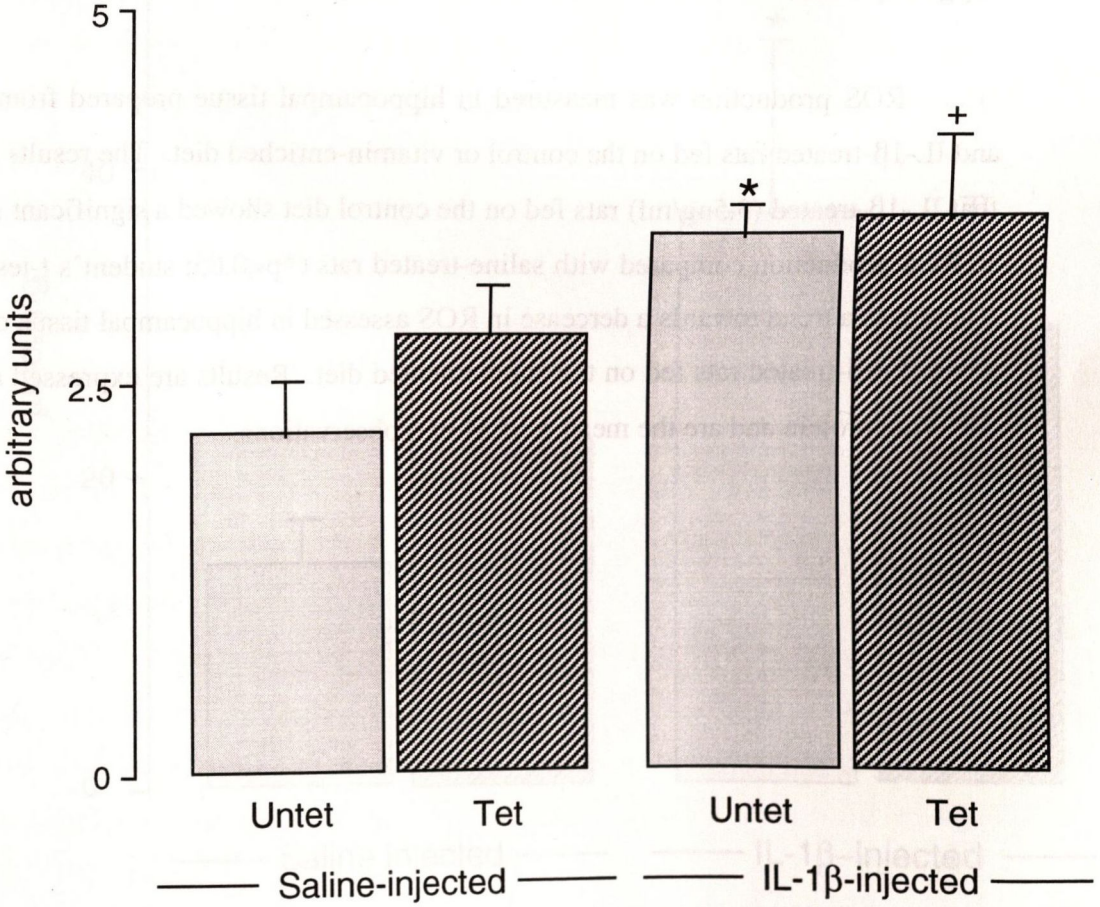

5
2.5
0
arbitrary units
*
+
Untet
Tet
Untet
Tet
Saline-injected
IL-1β-injected

Fig. 4.5 Effect of IL-1β-treatment and dietary supplementation on reactive oxygen species:

ROS production was measured in hippocampal tissue prepared from saline- and IL-1β-treated rats fed on the control or vitamin-enriched diet. The results indicate that IL-1β-treated (3.5ng/ml) rats fed on the control diet showed a significant increase in ROS production compared with saline-treated rats (*$p<0.05$; student's t-test; n=6). There was a trend towards a dercease in ROS assessed in hippocampal tissue obtained from IL-1β-treated rats fed on the supplemented diet. Results are expressed as μmol DCF/mg protein and are the mean ± SEM of 6 observations.

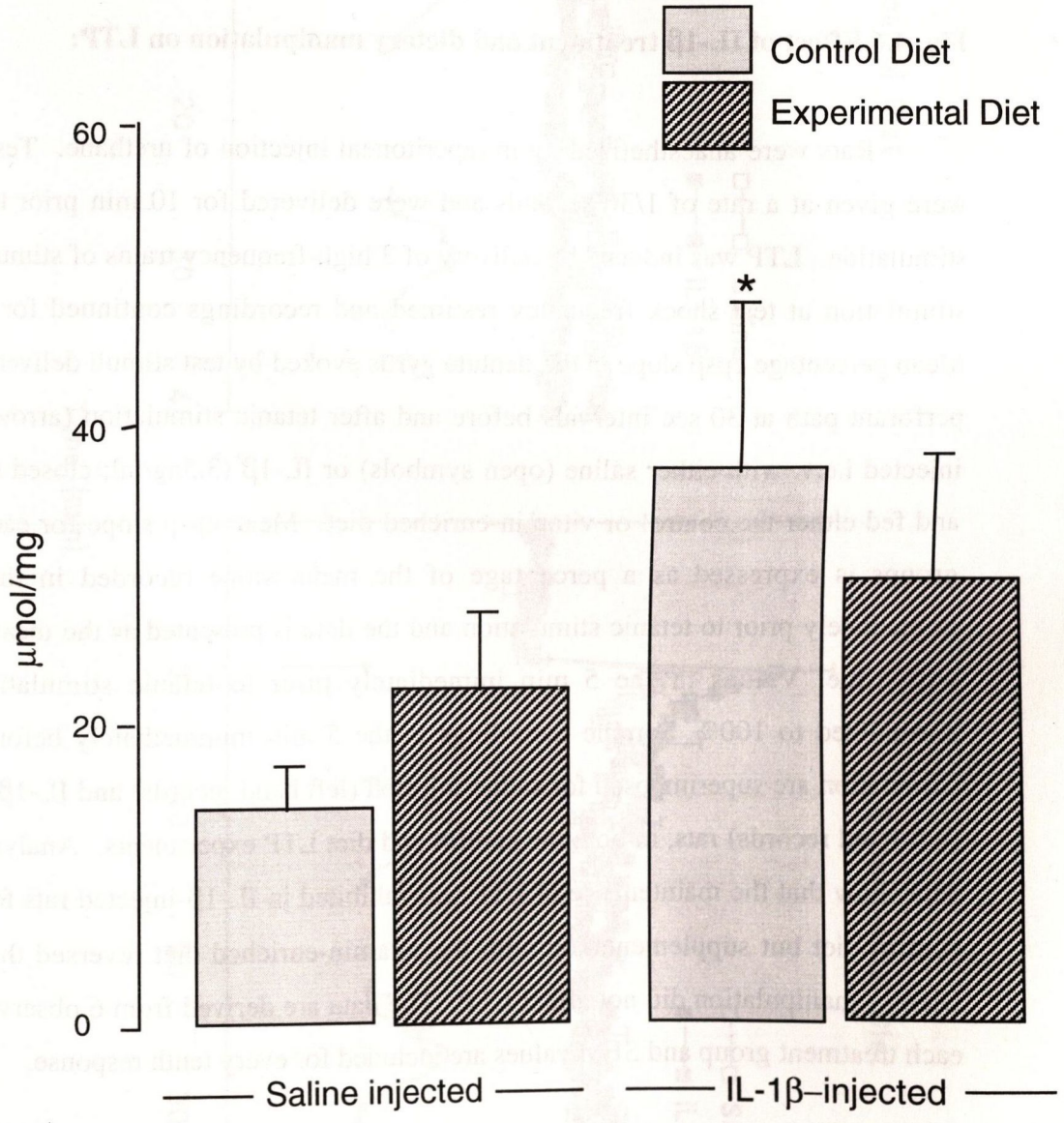
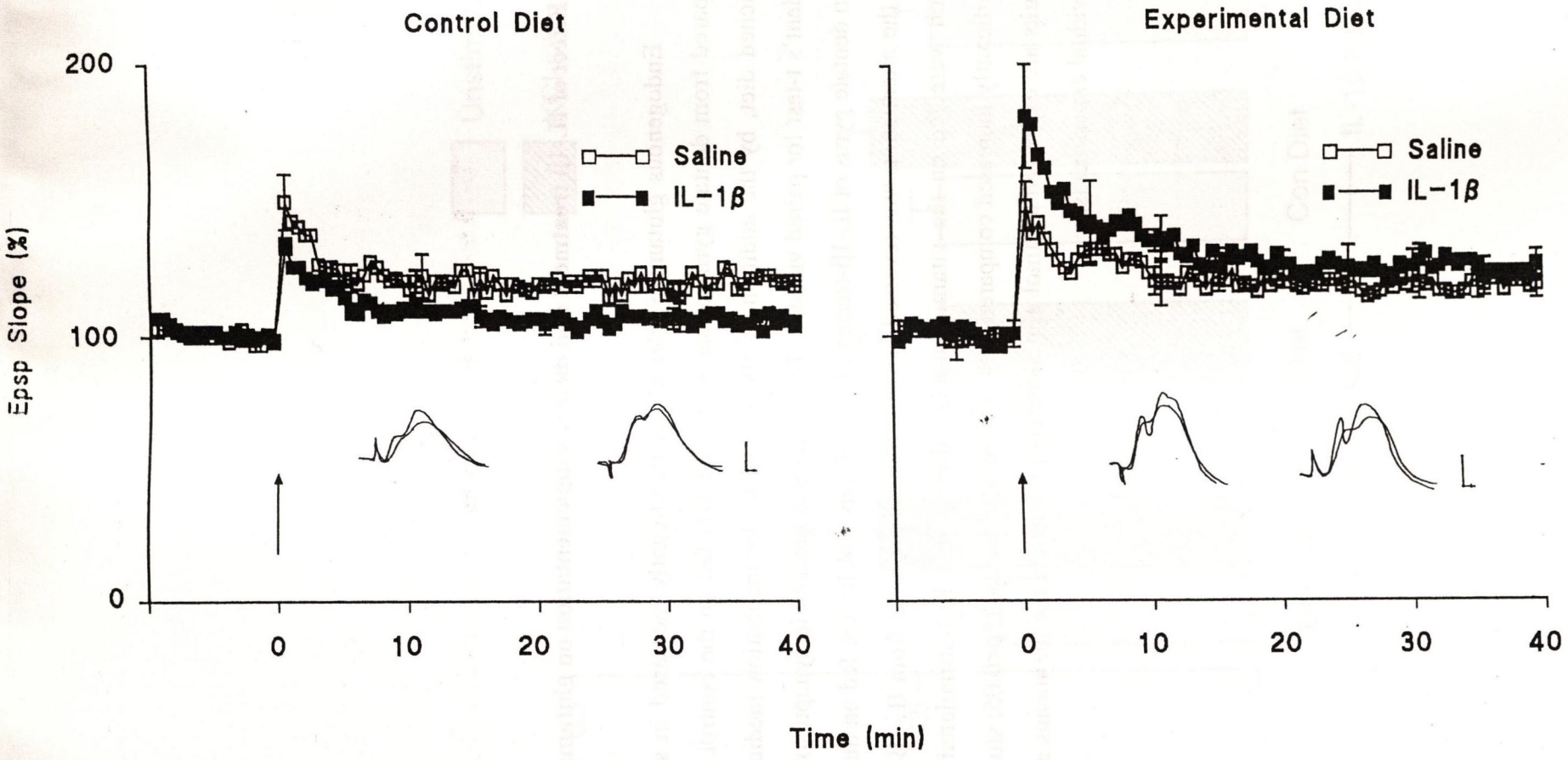

Control Diet
Experimental Diet
60
40
20
0
µmol/mg
*
Saline injected
IL-1β–injected

Fig. 4.6 Effect of IL-1β treatment and dietary manipulation on LTP:

Rats were anaesthetized by intraperitoneal injection of urethane. Test shocks were given at a rate of 1/30 seconds and were delivered for 10 min prior to tetanic stimulation. LTP was induced by delivery of 3 high-frequency trains of stimuli before stimulation at test shock frequency resumed and recordings continued for 45 min. Mean percentage epsp slope in the dentate gyrus evoked by test stimuli delivered to the perforant path at 30 sec intervals before and after tetanic stimulation (arrow) in rats injected i.c.v. with either saline (open symbols) or IL-1β (3.5ng/ml; closed symbols) and fed either the control or vitamin-enriched diet. Mean epsp slope for each of the groups is expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation and the data is presented as the mean change with time. Values in the 5 min immediately prior to tetanic stimulation were normalized to 100%. Sample recordings in the 5 min immmediately before tetanic stimulation are superimposed for saline-injected (left hand records) and IL-1β-injected (left hand records) rats, in both the control and diet LTP experiments. Analysis of the data show that the maintenance of LTP was inhibited in IL-1β-injected rats fed on the control diet but supplementation with the vitamin-enriched diet reversed this effect. Dietary manipulation did not affect LTP. The data are derived from 6 observations in each treatment group and SEM values are included for every tenth response.


Control Diet
Experimental Diet
Saline
IL-1β
Saline
IL-1β
200
100
0
Epsp Slope (%)
0
10
20
30
40
0
10
20
30
40
Time (min)

4.7 Effect of IL-1β treatment and dietary supplementation on glutamate release:

Endogenous glutamate release was significantly increased in synaptosomes prepared from dentate gyrus of saline-injected rats fed on the control and vitamin-enriched diet, by the addition of 50mM KCl to the incubation medium (*$p<0.05$; student's t-test for paired vlaues). This effect was blocked in synaptosomes prepared from dentate gyrus of IL-1β-treated (3.5ng/ml) rats which were fed on the control diet, but the attenuation was reversed in synaptosomes prepared from IL-1β-injected rats which were fed on the vitamin-enriched diet so that KCl-stimulated release was significantly increased compared with unstimulated release (*$p<0.05$; student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of six individual experiments.

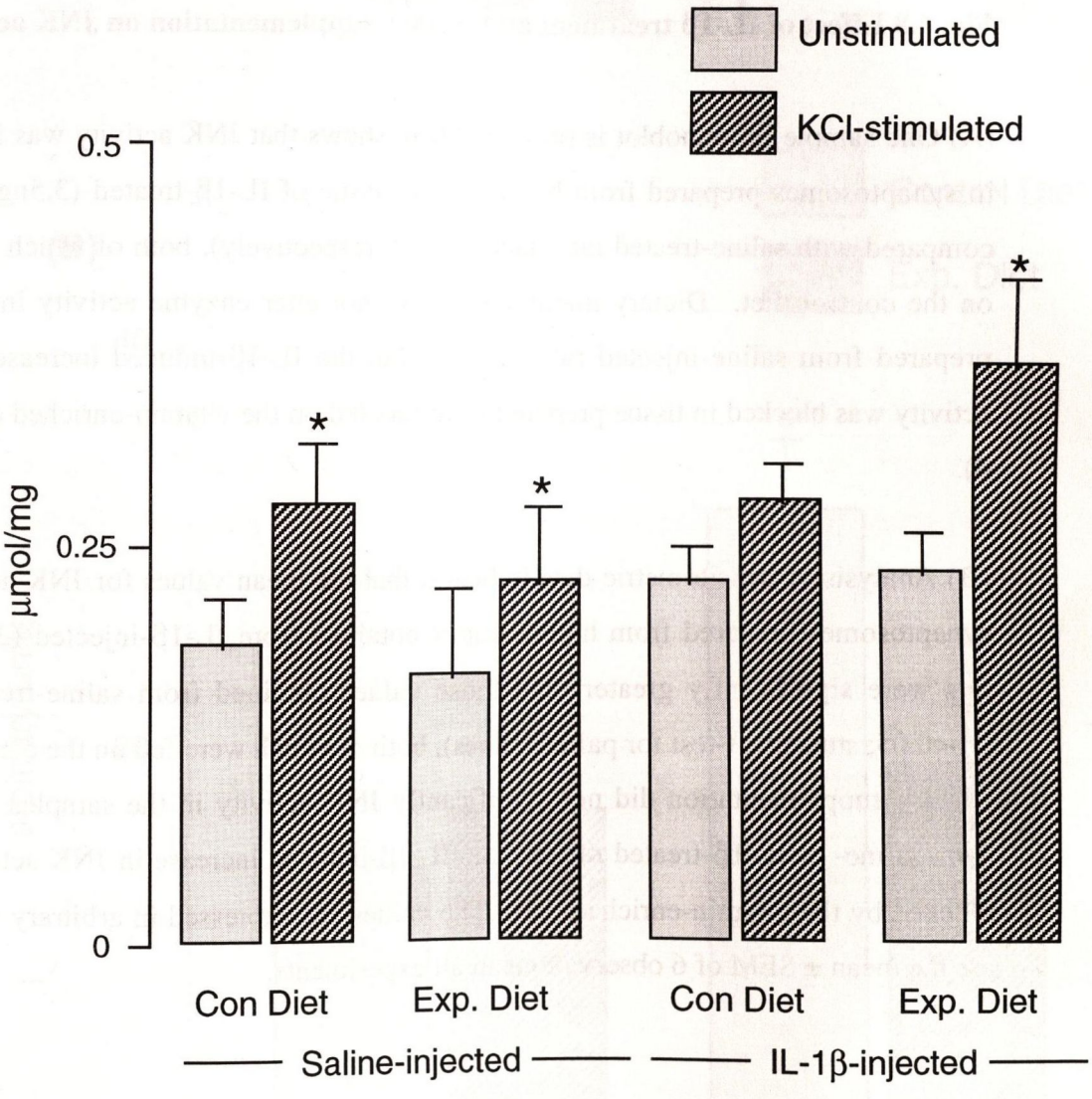

Unstimulated
KCl-stimulated
0.5
0.25
0
µmol/mg
*
*
*
Con Diet
Exp. Diet
Con Diet
Exp. Diet
Saline-injected
IL-1β-injected

Fig. 4.8 Effect of IL-1β treatment and dietary supplementation on JNK activity:

(A) One sample immunoblot is presented here shows that JNK activity was increased in synaptosomes prepared from hippocampal tissue of IL-1β-treated (3.5ng/ml) rats compared with saline-treated rats (lanes 1 & 3, respectively), both of which were fed on the control diet. Dietary manipulation did not alter enzyme activity in samples prepared from saline-injected rats (lane 2), but the IL-1β-induced increase in JNK activity was blocked in tissue prepared from rats fed on the vitamin-enriched diet (lane 4).

(B) Analysis of densitometric data indicates that the mean values for JNK activity in synaptosomes prepared from hippocampus obtained from IL-1β-injected (3.5ng/ml) rats were significantly greater than those values obtained from saline-treated rats (*$p<0.05$; student's t-test for paired values), both of which were fed on the control diet. Dietary supplementation did not significantly JNK activity in the samples prepared from saline- or IL-1β-treated rats but the IL-1β-induced increase in JNK activity was blocked by the vitamin-enriched diet. The values are expressed in arbitrary units and are the mean ± SEM of 6 observations in all experiments.

p

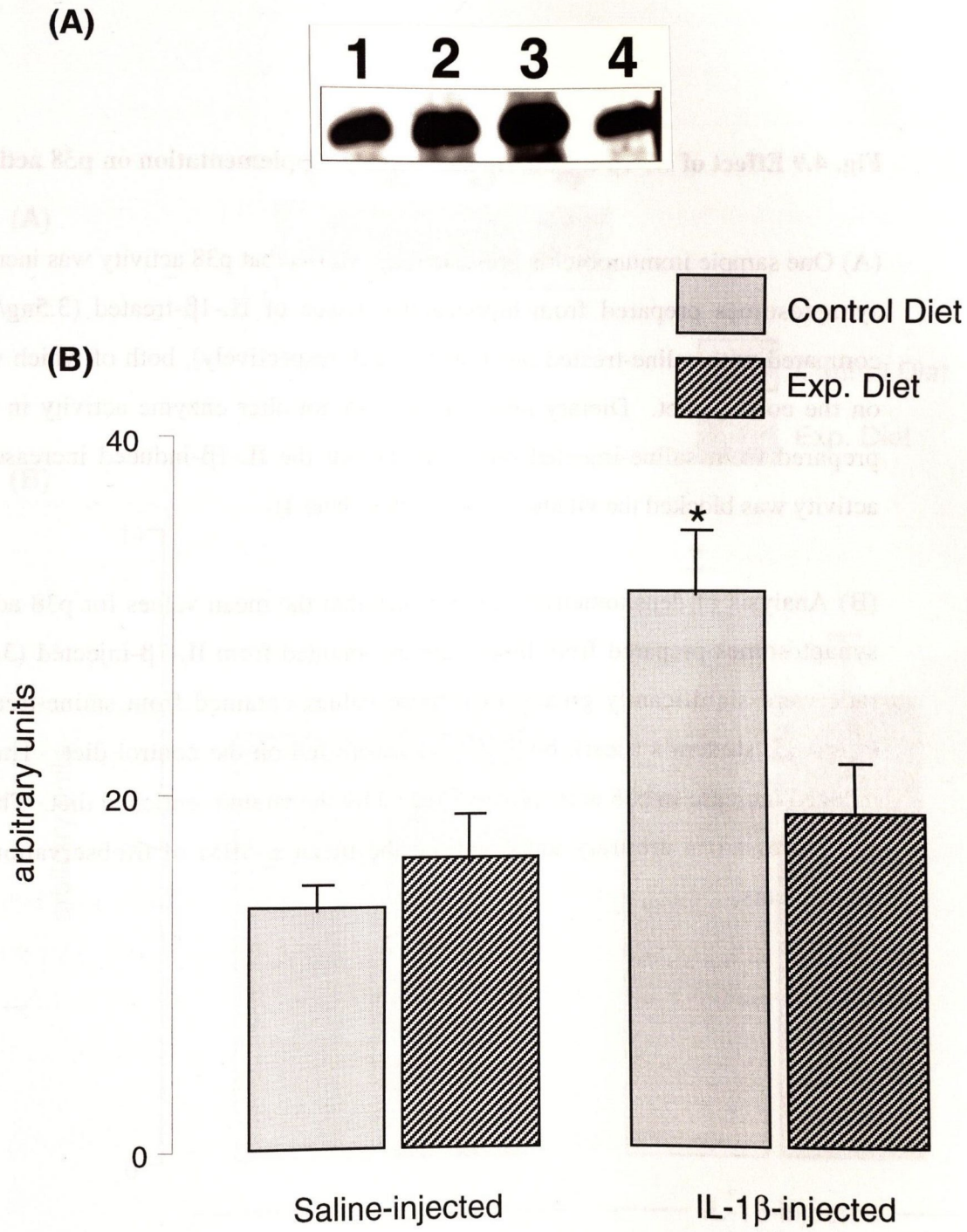
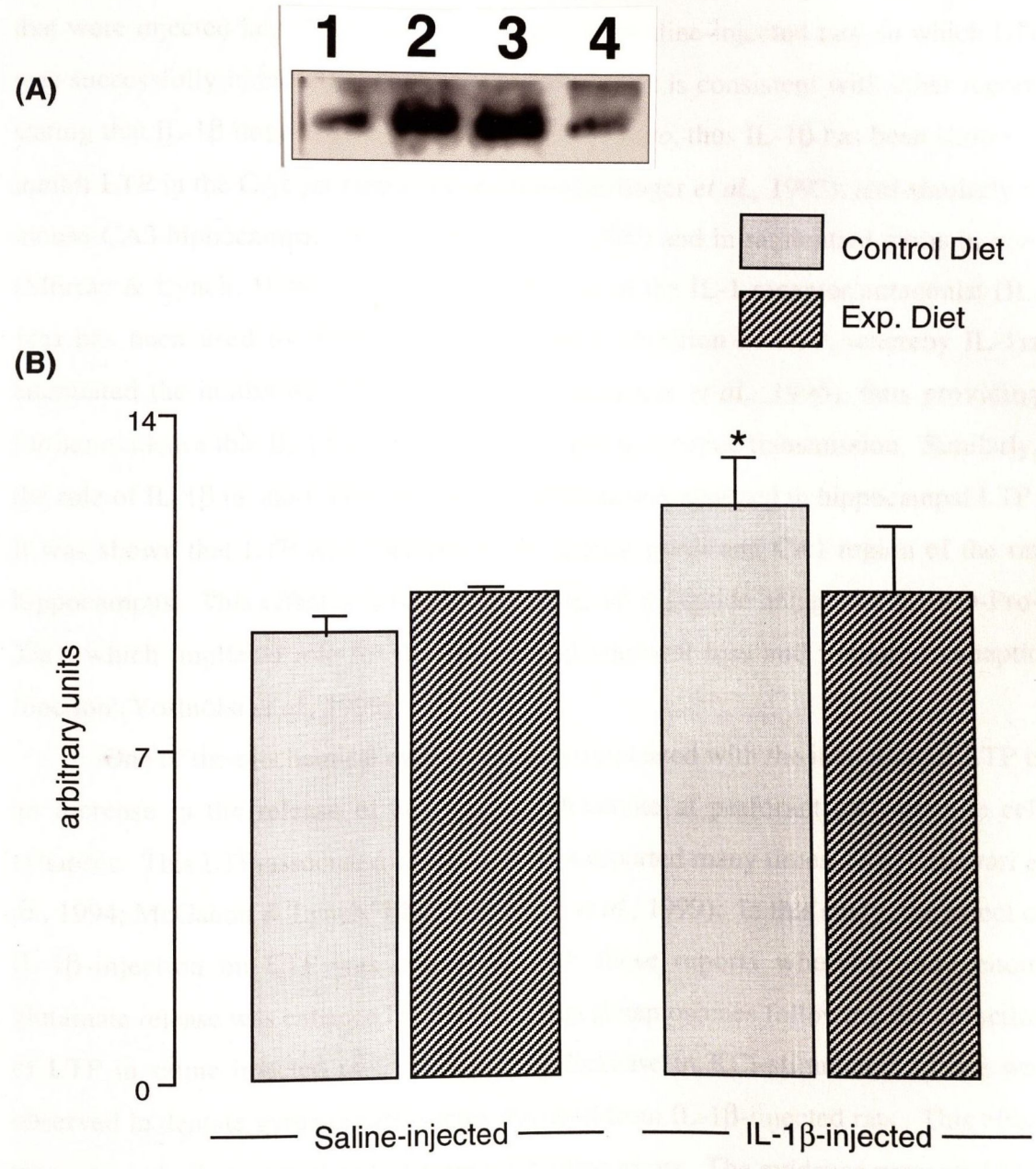

(A)
1 2 3 4
(B)
Control Diet
Exp. Diet
40
20
0
arbitrary units
*
Saline-injected
IL-1β-injected

Fig. 4.9 Effect of IL-1β treatment and dietary supplementation on p38 activity:

(A) One sample immunoblot is presented here shows that p38 activity was increased in synaptosomes prepared from hippocampal tissue of IL-1β-treated (3.5ng/ml) rats compared with saline-treated rats (lanes 1 & 3, respectively), both of which were fed on the control diet. Dietary manipulation did not alter enzyme activity in samples prepared from saline-injected rats (lane 2), but the IL-1β-induced increase in p38 activity was blocked the vitamin-enriched diet (lane 4).

(B) Analysis of densitometric data indicates that the mean values for p38 activity in synaptosomes prepared from hippocampus obtained from IL-1β-injected (3.5ng/ml) rats, were significantly greater than those values obtained from saline-treated rats (*$p<0.05$; student's t-test), both of which were fed on the control diet. The IL-1β-induced increase in p38 activity was blocked by the vitamin-enriched diet. The values are expressed in arbitrary units and are the mean ± SEM of 6 observations in all experiments.


(A)
1 2 3 4
Control Diet
Exp. Diet
(B)
14
7
0
arbitrary units
*
Saline-injected
IL-1β-injected

4.4 Discussion

The objectives of this study were firstly, to examine the effect of IL-1β injection on LTP in the rat dentate gyrus *in vivo* and to establish the effect this may have on glutamate release and the stress-activated protein kinases, JNK and p38. The second aim was to compare the effect of a short-term vitamin-enriched diet, consisting of vitamin E and vitamin C, with a control diet, on IL-1mediated neuronal changes.

The initial results suggested that LTP was impaired in the dentate gyrus of rats that were injected i.c.v. with IL-1β, compared with saline-injected rats, in which LTP was successfully induced and maintained. This result is consistent with other reports stating that IL-1β impairs LTP, both *in vivo* and *in vitro*, thus IL-1β has been shown to inhibit LTP in the CA1 rat hippocampal slices (Bellinger *et al.,* 1993), and similarly in mouse CA3 hippocampal slices (Katsuki *et al.,* 1990) and in rat dentate gyrus in vivo (Murray & Lynch, 1998a). Additionally, the use of the IL-1 receptor antagonist (IL-1ra) has been used to block the IL-1β-induced inhibition of LTP, whereby IL-1ra attenuated the inhibitory effect of IL-1β (Cunningham *et al.,* 1996), thus providing further evidence that IL-1β plays an inhibitory role in synaptic transmission. Similarly, the role of IL-1β in short-term incomplete ischemia was assessed in hippocampal LTP. It was shown that LTP was impaired in the dentate gyrus and CA1 region of the rat hippocampus. This effect was blocked by an IL-1β tripeptide antagonist (Lys-D-Pro-Thr), which implies a role for IL-1β-mediated neuronal loss and impaired synaptic function (Yoshioka *et al.,* 1999).

One of the biochemical events primarily associated with the induction of LTP is an increase in the release of endogenous glutamate at perforant path-granule cell synapses. This LTP-associated change has been reported many times (e.g. Canevari *et al.*, 1994; McGahon & Lynch, 1996; McGahon *et al.,* 1999). In this study, the effect of IL-1β-injection on LTP was consistent with these reports whereby endogenous glutamate release was enhanced in dentate gyrus synaptosomes following the induction of LTP in saline-injected rats. However, a decrease in KCl-stimulated release was observed in dentate gyrus synaptosomes obtained from IL-1β-injected rats. This effect was seen in both tetanized and untetanized dentate gyrus. The evidence presented here therefore confirms previous reports that LTP in the dentate gyrus is tightly coupled with an increase in glutamate release. However, these data suggest that the IL-1β-

induced impairment in LTP was associated with an inhibitory effect on KCl-stimulated glutamate release.

The results also indicate that the inhibitory effect of IL-1β on LTP is associated with a stimulatory effect on the activities of JNK and p38. Although both JNK and p38 were unaffected by tetanic stimulation per se, the activity of both kinases was increased in untetanized tissue obtained from IL-1β-injected rats. The effect of IL-1β on JNK and p38 activity has also been established in other cell types. For example, the effect of IL-1β on JNK activity has been observed in human glomerular mesangial (Uciechowski *et al.,* 1996) and HeLa (Raingeaud *et al.,* 1995) cells. The IL-1β-induced effect on p38 activity has been also observed in similar situations. IL-1β-induced activation of p38 has been reported in Chinese hamster CC139 (Guay *et al.,* 1997) and HeLa (Raingeaud *et al.*, 1995) cells. The effect of IL-1β-injection on LTP and the resulting increase in JNK and p38 activity observed in untetanized dentate gyrus is consistent with the finding that SB 320580, an inhibitor of p38, has been shown to inhibit the IL-1β-induced inhibition of LTP in the rat dentate gyrus (Coogan *et al.,* 1999). Other studies have also shown that the effects of IL-1β are mediated through p38. For example, the role of p38 in IL-1β-induced growth inhibition of A375 melanoma cells was assessed by using SB 230580. The involvement of p38 in IL-1β-induced growth inhibition of these cell types was confirmed as the p38 inhibitor reversed the effect of IL-1β (Itoh *et al.,* 1999). Baldassare and co-workers (1999) have also reported that p38 is involved in mediating the effects of IL-1β. This study showed that the inhibitor of p38, SB 203580, inhibited IL-1β protein levels, mRNA levels, transcription of the IL-1β gene in a macrophage-like cell line, J774 cells.

It has been suggested that the underlying causes of IL-1β-induced changes might be as a result of an increase in the production of reactive oxygen species (ROS). Reportedly, IL-1β increases the production of ROS in the rat hippocampus and hydrogen peroxide (H_2O_2), a common oxidant involved in oxidative damage, can result in an increase in IL-1β concentration (O' Donnell *et al.,* 2000; Murray & Lynch, 1998a). In conjunction with these findings, it is known that UV radiation (Zhang *et al.,* 1997) and osmotic stress (Qin *et al.,* 1999), both of which can induce the formation of ROS, increase the activity of both kinases in cultured HeLa cells, *in vitro* (Raingeaud *et al.,* 1995). To further support the theory that ROS, IL-1β and

stress-activated protein kinases are inter-connected, it has also been shown that treatment of HeLa cells with H_2O_2 induces apoptosis and activates JNK and p38 (Wang *et al.,* 1998). Similarly in primary cultures of astrocytes, H_2O_2-induced activation of JNK was observed (Tournier *et al.,* 1997). These reports, and the results presented here, suggest that an increase in the concentration of IL-1β may induce an increase in the production of ROS, which consequently activates the stress-activated protein kinases, thus blocking the expression of LTP.

Further findings in this study also show that the production of ROS was increased in hippocampal tissue obtained from IL-1β-injected rats fed on the control diet. This evidence implies that IL-1β-treated neuronal tissue was exposed to oxidative stress. In contrast to this finding, the effect of IL-1β-injection on ROS assessed in hippocampal tissue obtained from rats fed on the vitamin-enriched diet was reversed, indicating that the antioxidant diet had a significant role in sequestering ROS. These findings are supported by other reports which state that dietary supplementation downregulates the production of ROS. Dietary restriction in aged rats caused a decrease in the production of ROS compared with rats fed *ad lib* (Choi & Yu, 1995). It has been established that the production of ROS is increased in aged rats and this aged-related increase in ROS is reversed by a long-term (3 months) diet supplementation with vitamin E and C (Murray & Lynch, 1998). This result, and reports from other groups, support the hypothesis that ROS plays a fundamental role in impairment of synaptic function, particularly in the hippocampus.

To further investigate this theory, dietary supplementation was used. Vitamins E and vitamin C were incorporated into the daily diet, in an attempt to reverse the effects of IL-1β-injection on the expression of LTP, KCl-stimulated glutamate release and the activities of JNK and p38, by interacting with ROS. The data presented here show that the IL-1β-induced inhibition of LTP was blocked by dietary manipulation, suggesting that the inhibitory effect of IL-1β may be mediated through ROS production within the hippocampus. This result is consistent with the finding that H_2O_2, which increases

ROS, inhibited LTP in guinea pig CA1 *in vitro* (Pellmar *et al.,* 1991). It was also established that H_2O_2 reduces excitatory post synaptic potentials in the CA1 region of the guinea pig hippocampus (Pellmar, 1997). Furthermore, it has been reported that superoxide, a ROS (Halliwell, 1992) inhibits LTP in area CA1 of rat

hippocampal slices (Klann *et al.*, 1998). Low concentrations of H_2O_2 were reported to block the induction of non-NMDA associated LTP. More importantly, in this study, it was shown that in aged rats, where the production of H_2O_2 is more profound, non-NMDA-dependent tetanic LTP was markedly attenuated (Auerbach & Segal, 1997). When catalase was applied in this situation to break down H_2O_2, the H_2O_2-induced inhibition of this type of LTP was reversed (Aubersach & Segal, 1997).

In parallel with the analysis of LTP, the effect of IL-1β-injection on KCl-stimulated glutamate release in rats fed on the control and vitamin-enriched diet, was assessed. The data obtained from this experiment showed that the inhibitory effect of IL-1β on glutamate release was blocked by dietary supplementation. Consistent with this result, it has been shown that the age related inhibition of KCl-stimulated glutamate release in rat dentate gyrus synaptosomes, was reversed by a diet enriched with docosahexanoic acid, a polyunsaturated fatty acid necessary for the maintenance of membrane fluidity (McGahon *et al.*, 1999). Similarly, aged rats fed on a vitamin-enriched diet had the ability to sustain LTP in the dentate gyrus *in vivo* (Murray & Lynch, 1998a). From these findings, it was hypothesized that the inability of aged rats to sustain LTP may be triggered by an increase in lipid peroxidation, a common characteristic of tissue displaying oxidative damage (Gupta *et al.*, 1991), an increase of which is associated with aging (Murray & Lynch, 1998a). O' Donnell and Lynch (1998) have also presented evidence that dietary antioxidant supplementation reverses age-related neuronal changes such as increased IL-1β concentration, decreased concentrations of vitamin E and C and both increased in lipid peroxidation observed in entorhinal cortical tissue.

Coupled with the IL-1β-induced inhibition of glutamate release, was an increase in the activity of JNK and p38, both of which were attenuated with dietary supplementation. The data show that the effect of IL-1β-injection on tissue prepared from rats fed on the control diet resulted in an increase in JNK and p38 activity. An IL-1β-induced increase in JNK and p38 activity in the hippocampus confirms the findings of other groups, in which an IL-1β-induced increase in JNK and p38 is activated in various cell types (Raingeaud *et al.*, 1995; Guay *et al.*, 1997). These IL-1β-stimulated changes in release and stress-activated protein kinases were inhibited in tissue prepared from rats which were fed on the vitamin-enriched diet. It could be hypothesised that this attenuation of the effect of IL-1β on JNK and p38 activity is as

a result of an increase in anti-oxidant concentration in the hippocampus, thereby decreasing the ROS/IL-1β-induced activity of JNK and p38.

It could be proposed that ROS mediates the IL-1β-induced activation of JNK and p38 activity, resulting in inmpaired glutamate release and expression of LTP. This hypothesis was strengthened by previous reports whereby UV radiation (Zhang *et al.,* 1997) and osmotic stress (Qin et al., 1999), which have the ability to induce the formation of ROS, stimulate these stress-activated protein kinases in HeLa cells (Raingeaud *et al.,* 1995). Further evidence has suggested that the activation of JNK, but not p38, may be mediated by ROS, such that these UV responses are mediated by ROS, as intracellular glutathione, an antioxidant, was diminished by these responses (Devary, *et al.,* 1992).

The results of this study suggest the effect of IL-1β-injection on LTP is inhibitory, due to an IL-1β-induced increase in the activity of the stress-activated protein kinases, JNK and p38. The data are consistent with the hypothesis that that JNK and p38 exert an inhibitory effect on endogenous glutamate release. They further suggest that the effects are a consequence of an IL-1β-induced increase in ROS formation, since all the effects of IL-1β are inhibited by dietary manipulation with antioxidant vitamins.

Chapter 5

Analysis of the effects of stress-activated protein kinases and interleukin-1β with age: The role of dietary supplementation

5.1 Introduction

One of the most significant effects of aging is impaired cognitive function and synaptic plasticity (Barnes, 1979). Various groups have established that aged rats show an impaired ability to sustain LTP, such as Landfield and colleagues (1978) who demonstrated the inability of aged rats to sustain LTP in the hippocampus and suggesting a correlation with age-related behavioural problems. This report is supported by more recent findings that aged rats failed to sustain LTP rat dentate gyrus *in vivo* (Barnes, 1979; McGahon *et al.*, 1997; Murray & Lynch, 1998) and the ability of aged rats to sustain Ca^{2+} -induced LTP in CA1 hippocampal slices *in vitro* (Diana *et al.*, 1995).

It has been recently reported that impaired LTP in rat dentate gyrus is coupled with an increase in the concentration of the proinflammatory cytokine, interleukin-1β (IL-1β), in the hippocampus (Murray & Lynch, 1998). Thus it was demonstrated that an increase in the concentration of IL-1β was evident in aged rats and stressed rats, both of which were unable to sustain LTP (Murray & Lynch, 1998); these data suggest a negative correlation between IL-1β and LTP. An IL-1β-induced impairment in LTP *in vitro* has been reported in mossy-fiber-CA3 pathway of the mouse hippocampus (Katsuki *et al.*, 1990), in the rat dentate gyrus (Cunningham *et al.*, 1996) and in the CA1 region of the rat hippocampus (Bellinger *et al.*, 1993). These reports suggest that the IL-1β-induced impairment in LTP and the increase in the concentration of IL-1β in the rat dentate gyrus, may be characteristic of degenerative changes associated in the aged rat brain (Murray & Lynch, 1998).

One suggested reason for IL-1β-induced neuronal impairments such as impaired LTP is that IL-1β induces an increase in reactive oxygen species (ROS) production (Murray & Lynch, 1998; O' Donnell *et al.*, 2000). An increase in ROS production can result in oxidative stress and neurological impairments and has been shown by various groups to have deleterious effects on synaptic functions such as LTP (Auerbach & Segal, 1997; Klann *et al.*, 1998). Reportedly, ROS increases the concentration of IL-1β in rat hippocampal tissue but IL-1β has been shown to increase the production of ROS, which suggests that a positive feedback loop exists (O' Donnell *et al.*, 2000). Studies have shown that ROS production is increased in aged rats and is associated with an increase in lipid peroxidation and a decrease in concentration of the polyunsaturated fatty acid, arachidonic acid (AA) (Murray and

Lynch, 1998a; O' Donnell & Lynch, 1998). The importance of arachidonic acid in LTP in aged animals has been investigated and has been proposed as a possible retrograde messenger in later stages of LTP (Bliss *et al.,* 1989). McGahon and collegues (1997) have demonstrated that dietary manipulation with AA reverses the age-related decrease in AA, enabling aged rats to sustain LTP, emphasising a role for AA.

An increase in IL-1β concentration and ROS triggers many cellular events, such as activation of stress-activated protein kinases, JNK and p38. Both stress-activated protein kinases are activated by environmental stresses including oxidative stress (Davis, 1995; Raingeaud *et al.*, 1995; Uceichowski *et al.,* 1996; Junger *et al.,*1997). Various studies have observed IL-1β-induced activation of JNK and p38 in cultured and circulating cells (Derijard *et al.,* 1994; Raingeaud *et al.,* 1995; Rizzo-Carlo-Stella *et al.,* 1996; Uceichowski *et al.,* 1996; Lu *et al.,* 1997). However, there is little evidence to suggest similar changes occur with age.

The results presented in the previous chapters suggest a negative correlation between the induction of LTP and IL-1β, whereas IL-1β appears to be positively correlated with JNK and p38. To further study this hypothesis, the aim of this study was to assess parallel age-related changes on LTP, IL-1β concentration and the activities of JNK and p38.

5.2 Methods

5.2.1 Animals

Male adult Wistar rats were housed as described in section 2.2.1. These rats of mean age 4 months (250-350g) or 22 months (450-550g) were used in these experiments. A total of 7 young and 13 aged rats were used in these studies. As part of this study, young and aged rats were fed for 8 weeks on a control diet and a diet enriched in 10mg docosahexanoic acid, 26% w/v in tuna oil. Isocaloric intake was assured by providing the control group with standard laboratory chow to which corn oil was added (section 2.2.2.).

5.2.2 Induction of LTP *in vivo*

Aged and young rats were anaesthetized by intraperitoneal (i.p.) injection and, once an adequate level of anaesthesia was achieved, a window of skull was removed in order to allow the placement of the stimulating and recording electrodes (see section 2.5). Test shocks (average stimulus strength 4.14 ± 0.15 V and 3.96 ± 0.12 V in young and aged rats, respectively) were given at 30 sec intervals for 10 min before and 40 min after tetanic stimulation (three trains of stimuli; 250Hz for 200 msec; 30 sec intervals). The second part of this study involved two subgroups of young and aged rats fed on a control and docosahexanoic acid diet. LTP was assessed in young rats fed on the control and supplemented diet and also on aged rats fed on the control and supplemented diet (I would like to acknowledge Dr. Bernie McGahon for her kind donation of the tissue obtained from the LTP experiments). The method for the induction of LTP in these subgroups was identical to the above protocol (see section 2.5). Results were expressed as the mean % increase in epsp slope measured against time.

5.2.3 Analysis of IL-1β concentration

The concentration of IL-1β was assessed in hippocampal homogenate obtained from young and aged rats. Additionally, IL-1β was measured in hippocampal homogenate obtained from young and aged rats, fed on the control and docosahexanoic acid diet. Hippocampal slices obtained from these groups were prepared as described in section 2.3.1. The slices were homogenized in lysis buffer (500µl; 25mM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml

pepstatin) and then protein concentration was equalized for each sample (section 2.4). Briefly, a 96-well microtiter plate was coated with capture antibody and standards (range: 0-1000pg/ml) or samples were incubated for 1 h prior to the addition of the secondary antibody. The detection antibody reagent and the working substrate, TMB, were added, and the reaction was stopped by the addition of 2N H_2SO_4 and the samples were read at 450nm (section 2.9). The concentration of IL-1β was expressed as pg IL-1β/mg protein/wet weight, corrected for protein concentration in this case as the study involved aged rats.

5.2.4 Analysis of JNK and p38 activity

The activity of JNK and p38 was assessed in hippocamapal tissue prepared from young and aged rats, and also in young and aged rats fed on the control and docosahexanoic diet. In both studies, hippocampal tissue was prepared from frozen slices (section 2.3.1.). The synaptosomal P_2 preparation was prepared as described in section 2.8.1. The prepared samples were loaded onto polyacrylamide gels to separate proteins which were transferred onto nitrocellulose paper and immunoblotted with either anti-JNK or anti-p38 antibodies, using the concentrations previously described in section 2.8.3. The antibody-protein complex was visualized using ECL detection, protein bands were quantified by densitometric analysis, and results were expressed as arbitrary units.

5.3 Results

5.3.1 Effect of age on LTP

Analysis of the data indicates that LTP was attenuated in aged rats compared with young rats (see fig. 5.1). The mean percentage epsp slopes in the 2 min immediately following tetanic stimulation (compared with the 5 min value immediately prior to tetanic stimulation) were 153.02 ± 3.85 % and 116.45 ± 2.72 % in the young and aged rats, respectively. The mean percentage epsp slope in the last 5 min of the experiment were 136.45 ± 2.15 % and 110.35 ± 2.08 %. These data indicate that there was a significant age-related decrease in both the early and persistent response to tetanic stimulation ($p<0.05$; student's t-test; n=7 and n=13 for young and aged rats, respectively).

5.3.2 Analysis of IL-1β concentration in the hippocampus of young and aged rats

IL-1β concentration was assessed in hippocampal tissue prepared from young and aged rats. Fig 5.2 indicates that IL-1β concentration was significantly increased in hippocampal tissue obtained from aged rats compared with young rats (*$p<0.05$; student's t-test). The mean values were 1.42 ± 0.10 pg/mg (SEM; n=7) and 2.58 ± 0.25 pg/mg (SEM; n=13) for young and aged rats, respectively.

5.3.3. Analysis of JNK activity in the hippocampus of young and aged rats

The activity of JNK was assessed in tissue prepared from hippocampal tissue obtained from young and aged rats using gel electrophoresis and immunoblotting with phosphospecific antibodies. One sample immunoblot indicates an-age related increase in JNK activity, see fig. 5.3 (A). An significant increase in mean JNK activity was seen in tissue prepared from aged rats compared with JNK activity assessed in hippocampal tissue obtained from young rats (*$p<0.05$; student's t-test; fig. 5.3 (B)). The results were quantified by densitometry and expressed in arbitrary units and the mean values were 34.2 ± 4.8 (SEM; n=6) and 47.6 ± 11.0 for JNK activity observed in hippocampal tissue of young and aged rats, respectively.

5.3.4 Analysis of p38 activity in the hippocampus of young and aged rats

The activity of p38 was assessed in tissue prepared from hippocampal tissue obtained from young and aged rats using gel electrophoresis and immunoblotting with phosphospecific antibodies. One sample immunoblot indicates an-age related increase in p38 activity, see Fig. 5.4 (A). A significant age-related increase in mean p38 activity was seen in tissue prepared from aged rats compared with p38 activity assessed in hippocampal tissue obtained from young rats (*$p<0.05$; student's t-test; Fig. 5.4 (B)). The results were quantified by densitometry and expressed in arbitrary units and the mean values were 1.09 ± 0.2 (SEM; n=6) and 2.01 ± 0.2 for p38 activity observed in hippocampal tissue of young and aged rats, respectively.

5.3.5 Effect of dietary supplementation on LTP in young and aged rats

The ability of aged and young rats to sustain LTP was assessed in rats fed on the control and docosahexanoic acid diet. Fig 5.7 indicates that there was a marked increase in the mean population epsp slope immediately following tetanic stimulation

in young rats fed on the control diet or the docosahexanoic acid diet. The mean percentage in the in the epsp slope in the 2 min immediately following tetanic stimulation was 153.02 ± 3.85 % and 132.76 ± 3.99 % in the control (n=6) and dietary (n=6) groups, assessed in young rats, respectively. In the last 5 min of the experiment the values were 128.9 ± 2.89 % and 126.86 ± 3.13 %, respectively. Fig 5.7 also reveals that age-related impairment in LTP was attenuated by dietary supplementation. The mean percentage change in epsp slope in the first 2 min after tetanic stimulation was 116.45 ± 2.75 %, and in the last 5 min of the experiment the value was 110.35 ± 2.08 % (n=4) which was significantly lower than the corresponding value observed in young rats ($p<0.05$; student's t-test). However, aged rats fed on the docosahexanoic acid diet showed an ability to sustain LTP which was similar to young rats. The mean percentage changes were 145.65 ± 3.54 % and 131.67 ± 3.59 % (SEM; n=8) in the first 2 min after tetanic stimulation and in the last 5 min of the experiment, respectively.

5.3.6. Effect of dietary supplementation on IL-1β concentration in the hippocampus of young and aged rats

The effect of dietary supplementation with docosahexanoic acid on IL-1β concentration was assessed in hippocampal tissue obtained from young and aged rats. The data illustrated in fig. 5.6. show that the concentration of IL-1β was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet compared to young rats fed on either diet (*$p<0.05$; student's t-test). The mean values were 2.17 ± 0.2 pg/mg (SEM; n=6) and 1.85 ± 0.4 pg/mg (SEM; n=4) for young rats fed on the control and docosahexanoic diet, respectively, and 3.4 ± 0.7 pg/mg (SEM; n=7) for aged rats fed on the control diet. The age-related increase in IL-1β concentration was markedly decreased in hippocampal tissue obtained from aged rats fed on the supplemented diet, compared with aged rats fed on the control diet, but this did not represent a statistically significant change. The mean value was 2.26 ± 0.3 pg/mg (SEM; n=9).

5.3.7 Effect of dietary supplementation on JNK activity in the hippocampus of young and aged rats

Fig. 5.7 (A) illustrates one sample immunoblot which reveals that JNK activity was increased in hippocampal tissue obtained from aged rats fed on the control diet (lane 1) compared with JNK activity assessed in hippocampal tissue obtained from young rats (lane 3). Additionally, JNK activity was decreased in hippocampal tissue obtained from aged rats, fed on the experimental diet (lane 4) compared with aged rats fed on the control diet. JNK activity was similar in hippocampus prepared from aged rats fed on the experimental diet and in young rats fed on both diets. Densitometric analysis, expressed as arbitrary units, revealed that JNK activity was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet, compared with young rats fed on the control diet (*$p<0.05$; student's t-test, analysis; fig. 5.8 (B)). The mean values were 1.07± 0.6 (SEM; n=3) and 1.43 ± 0.2 for JNK activity assessed in hippocampal tissue obtained from young and aged rats, fed on the control diet, respectively. JNK activity was significantly decreased in hippocampal tissue obtained from aged rats fed on the experimental diet compared to JNK activity observed in hippocampal tissue obtained from aged rats fed on the control diet, where the mean value was 0.84 ± 0.1 (+$p>0.05$; student's t-test; SEM; n=8). JNK activity assessed in hippocampal tissue obtained from young rats fed on either diet was similar to that of aged rats fed on the supplemented diet, where the mean value was 1.43 ± 0.2 (SEM; n=6).

5.3.8 Effect of dietary supplementation on p38 activity in the hippocampus of young and aged rats:

The effect of dietary manipulation was assessed on p38 activity in hippocampal tissue. Fig. 5.8 (A) illustrates one sample immunoblot which reveals that p38 activity was increased in hippocampal tissue obtained aged rats fed on the control diet (lane 3) compared with p38 activity assessed in hippocampal tissue obtained from young rats fed on the control diet (lane 1). Additionally, p38 activity was decreased in hippocampal tissue obtained from aged rats fed on the supplemented diet (lane4) compared with aged rats fed on the control diet (lane 3) such that the activity of p38 was similar to that observed in hippocampal tissue obtained from young rats fed on either diet (lane 1 and 2). Densitometric analysis, expressed as arbitrary units, revealed that p38 activity was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet, compared with young rats fed

on the control diet (*$p<0.05$; student's t-test; fi.g 5.10 (B)). The mean values were 3.4 ± 0.7 (SEM; n=3) and 6.2 ± 1.0 for p38 activity assessed in hippocampal tissue obtained from young and aged rats fed on the control diet, respectively. The decrease in p38 activity was not statistically significantly decreased in hippocampal tissue obtained from aged rats fed on the experimental diet were the mean value was 5.06 ± 0.8 (SEM; n=8). p38 activity assessed in hippocampal tissue obtained from young rats fed on the diet showed similar levels of activity to that of young rats fed on the control diet, where the mean values were 3.7 ± 1.5 (SEM; n=6).

Fig. 5.1 Effect of age on LTP

Rats were anaesthetized by intraperitoneal (i.p.) injection of urethane (1.5g/kg). Test shocks were delivered at a rate of 1/30 sec for 10 min prior to tetanic stimulation and then LTP was induced by delivery of 3 high frequency trains of stimuli (250Hz for 200 msec; at 30 sec intervals). Stimulation at test shock frequency resumed and recordings continued for a further 45 min. These data show that LTP is compromized in aged rats compared to young rats that have the ability to sustain LTP. Fig 5.1 shows the mean percentage epsp slope in the dentate gyrus evoked by test stimuli delivered to the perforant path at 30 sec intervals before and after tetanic stimulation in young (open symbols; n=7) and aged (closed symbols; n=13). The mean slope is expressed as a percentage of the slope recorded in the 5 min prior to tetanic stimulation and the data is expressed as the mean percentage change in epsp slope with time.

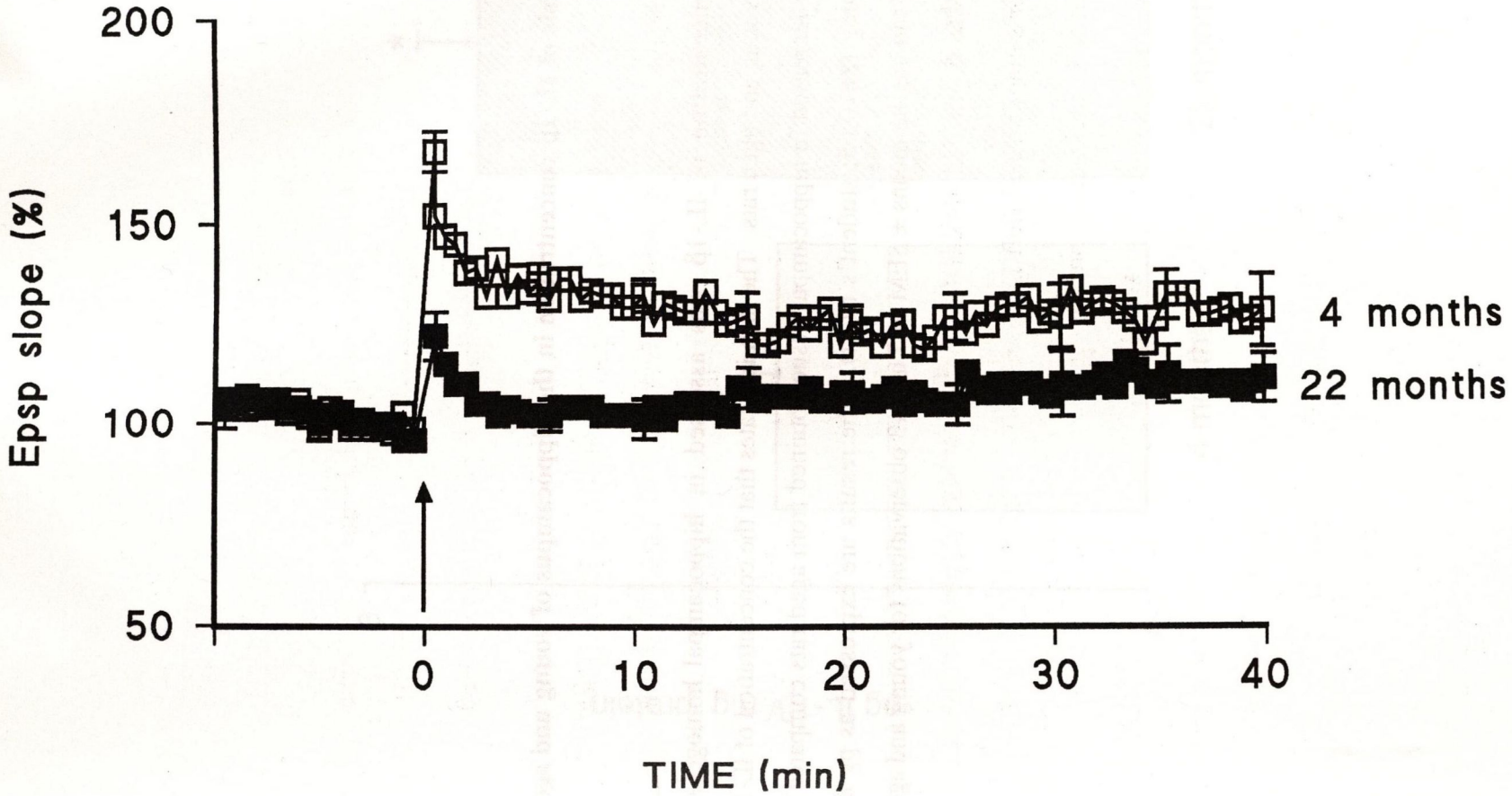

200
150
100
50
Epsp slope (%)
0
10
20
30
40
TIME (min)
4 months
22 months

Fig. 5.2 Analysis of IL-1β concentration in the hippocampus of young and aged rats:

The concentration of IL-1β was assessed in hippocampal homogenate obtained from young and aged rats. The data indicates that the concentration of IL-1β is significantly increased in hippocampal tissue obtained from aged rats compared to that of the young (*$p<0.05$; student's t-test). The results are expressed as pg IL-1β/mg protein and are the means ± SEM of 7 and 13 observations for young and aged rats, respectively.

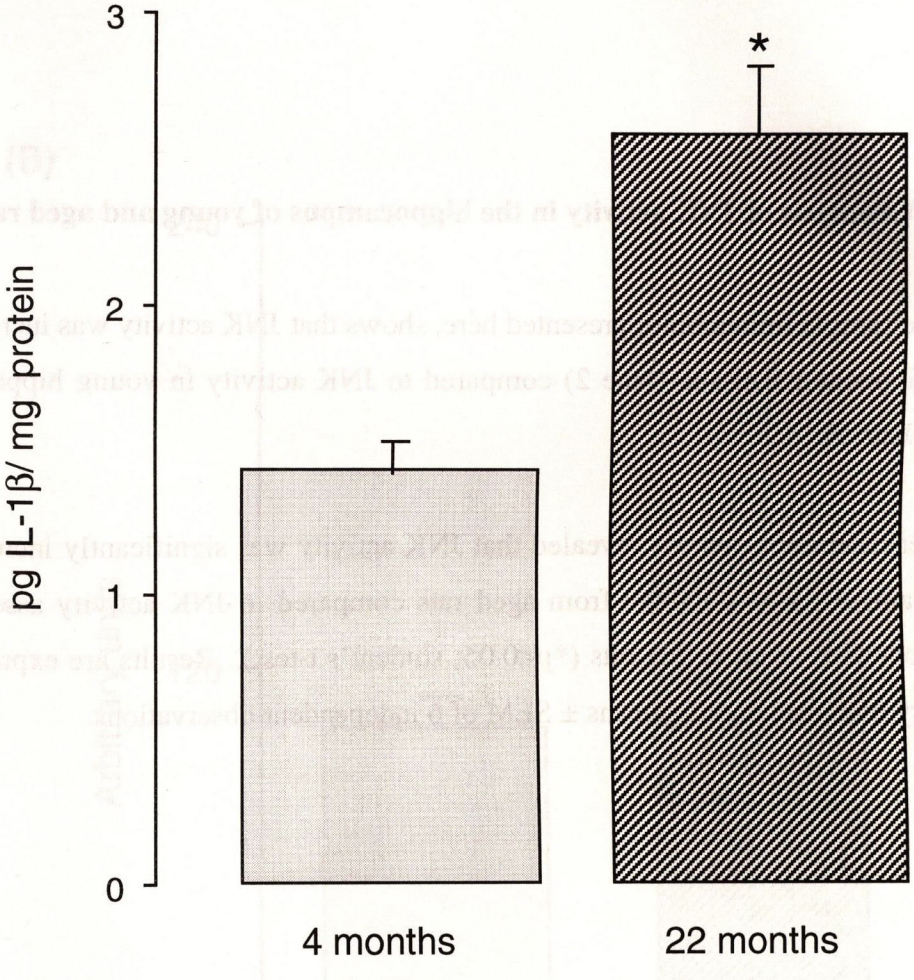
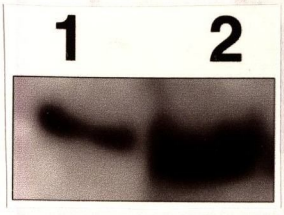

3
2
1
0
pg IL-1β/ mg protein
*
4 months
22 months

Fig. 5.3 Analysis of JNK activity in the hippocampus of young and aged rats:

(A) One sample immunoblot presented here, shows that JNK activity was increased in aged hippocampal tissue (lane 2) compared to JNK activity in young hippocampal tissue (lane 1).

(B) Densitometric analysis revealed that JNK activity was significantly increased in hippocampal tissue obtained from aged rats compared to JNK activity assessed in tissue obtained from young rats (*$p<0.05$; student's t-test). Results are expressed in arbitrary units and are the means ± SEM of 6 independent observations.

(A)



(B)

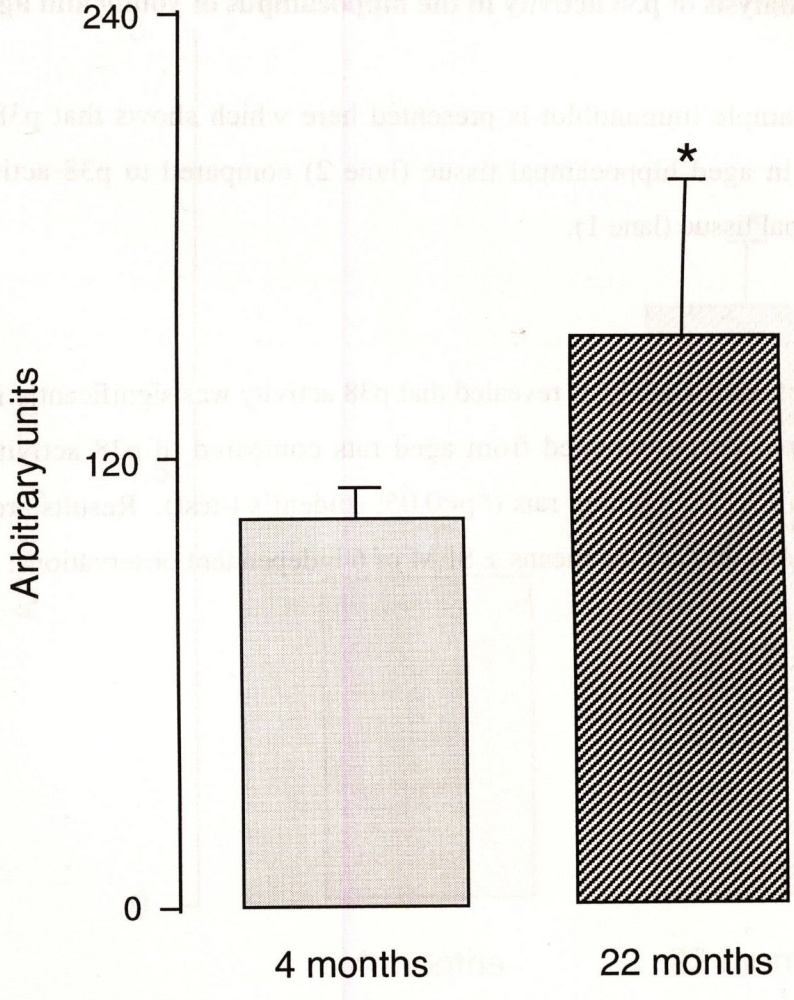
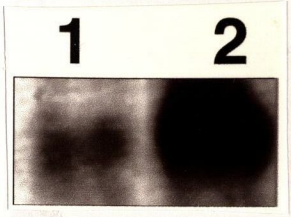


Fig. 5.4 Analysis of p38 activity in the hippocampus of young and aged rats:

(A) One sample immunoblot is presented here which shows that p38 activity was increased in aged hippocampal tissue (lane 2) compared to p38 activity in young hippocampal tissue (lane 1).

(B) Densitometric analysis revealed that p38 activity was significantly increased in hippocampal tissue obtained from aged rats compared to p38 activity assessed in tissue obtained from young rats (*$p<0.05$; student's t-test). Results are expressed in arbitrary units and are the means ± SEM of 6 independent observations.

(A)



(B)

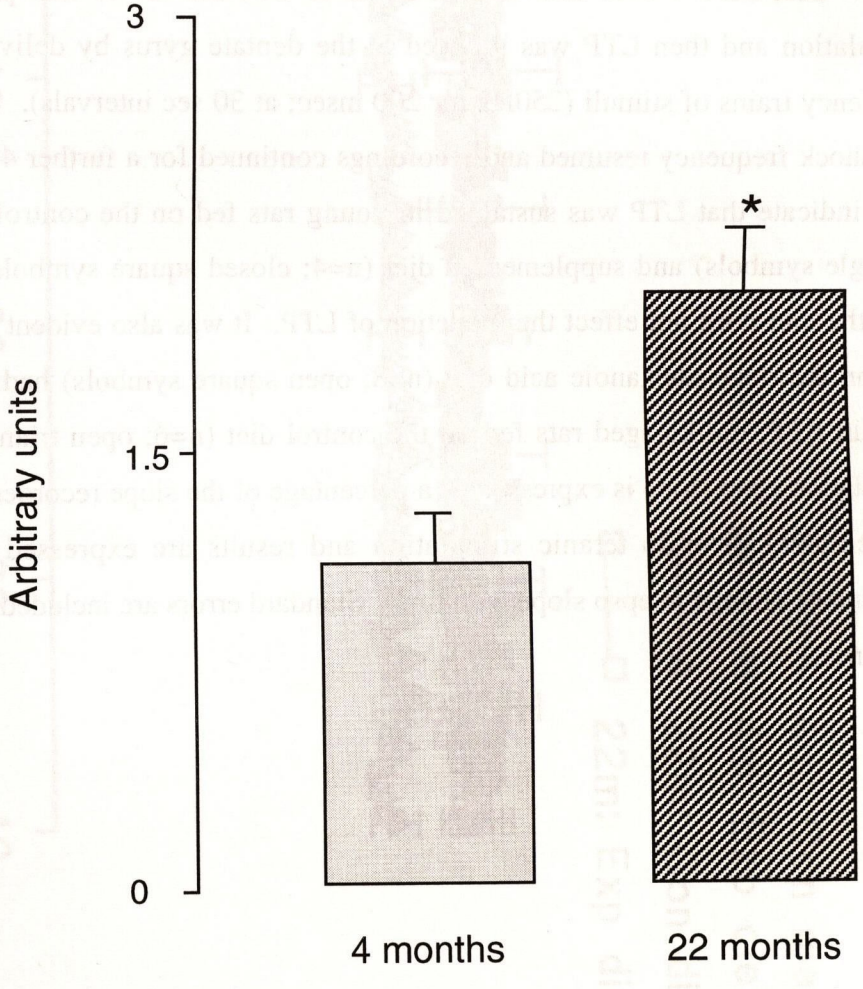
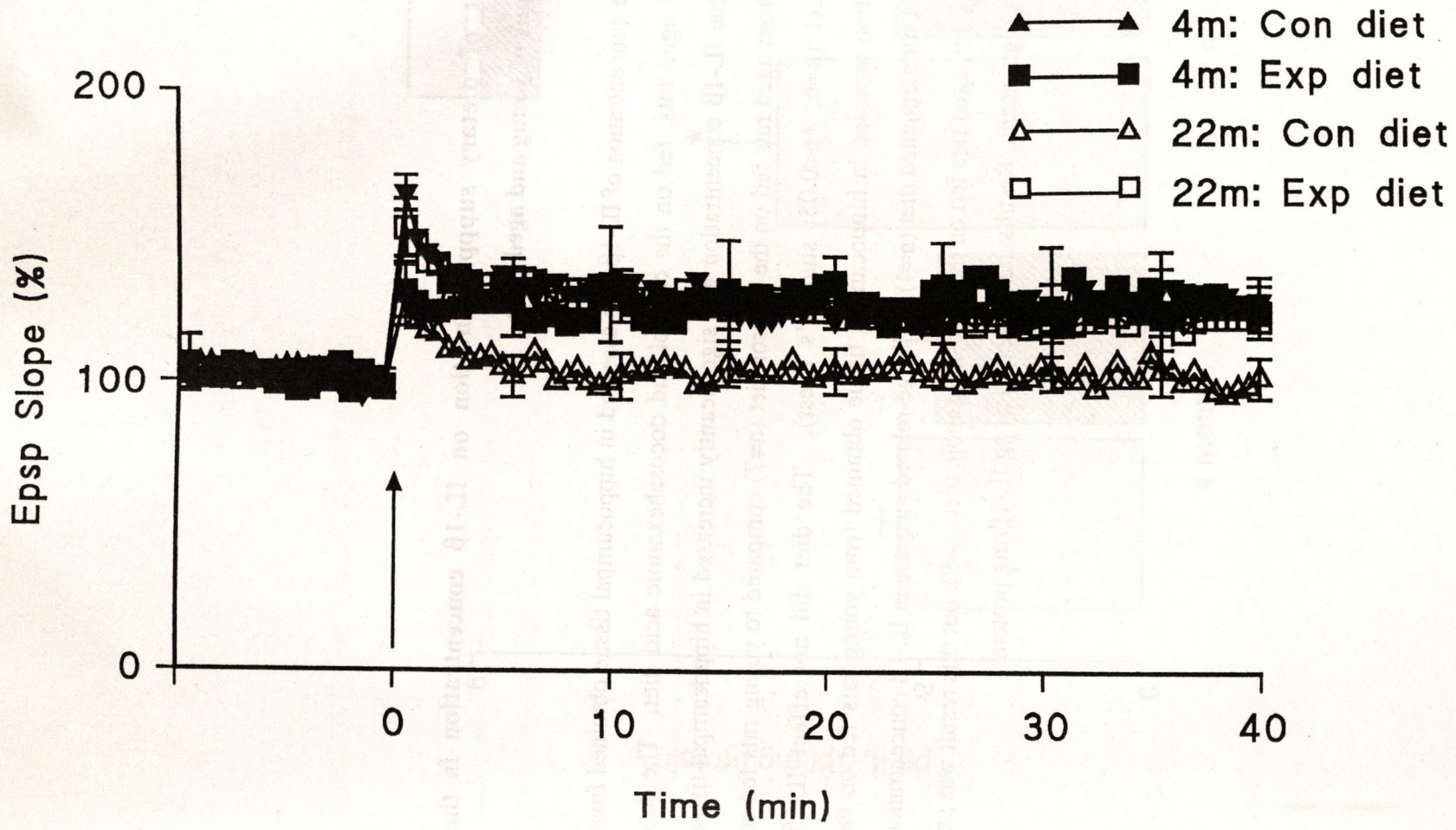


Fig. 5.5 Effect of dietary supplementation on LTP in young and aged rats:

Rats were anaesthetized with urethane by intraperitoneal (i.p.) injection (1.5 g/kg). Test shocks were delivered at a rate of 1/30 sec for 10 min prior to tetanic stimulation and then LTP was induced in the dentate gyrus by delivery of 3 high frequency trains of stimuli (250Hz for 200 msec; at 30 sec intervals). Stimulation at test shock frequency resumed and recordings continued for a further 45 min. These data indicate that LTP was sustained in young rats fed on the control (n=6; closed triangle symbols) and supplemented diet (n=4; closed square symbols), suggesting that the diet does not effect the induction of LTP. It was also evident that aged rats fed on the docosahexanoic acid diet (n=8; open square symbols) had the ability to sustain LTP, unlike aged rats fed on the control diet (n=6; open triangle symbols). Population epsp slope is expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and results are expressed as the mean percentage change in epsp slope with time. Standard errors are included for every 10^{th} response.


4m: Con diet
4m: Exp diet
22m: Con diet
22m: Exp diet
200
100
0
Epsp Slope (%)
0
10
20
30
40
Time (min)

5.6 Effect of dietary suppplementation on IL-1β concentration in the hippocampus of young and aged rats:

The concentration of IL-1β was assessed in hippocampal tissue obtained from young and aged rats, fed on the control and docosahexanoic acid diet. The data illustrate that IL-1β concentration was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet (n=7) compared to young rats fed on control diet (n=6; *$p<0.05$; student's t-test). The diet did not effect IL-1β concentration assessed in hippocampal tissue obtained from young rats fed on the control and supplemented diet (n=4). The age-related increase in IL-1β concentration was reversed in aged rats fed on the docosahexanoic diet (n=9), but this result was not statistically significant. Results are expressed as pg IL-1β/mg protein.

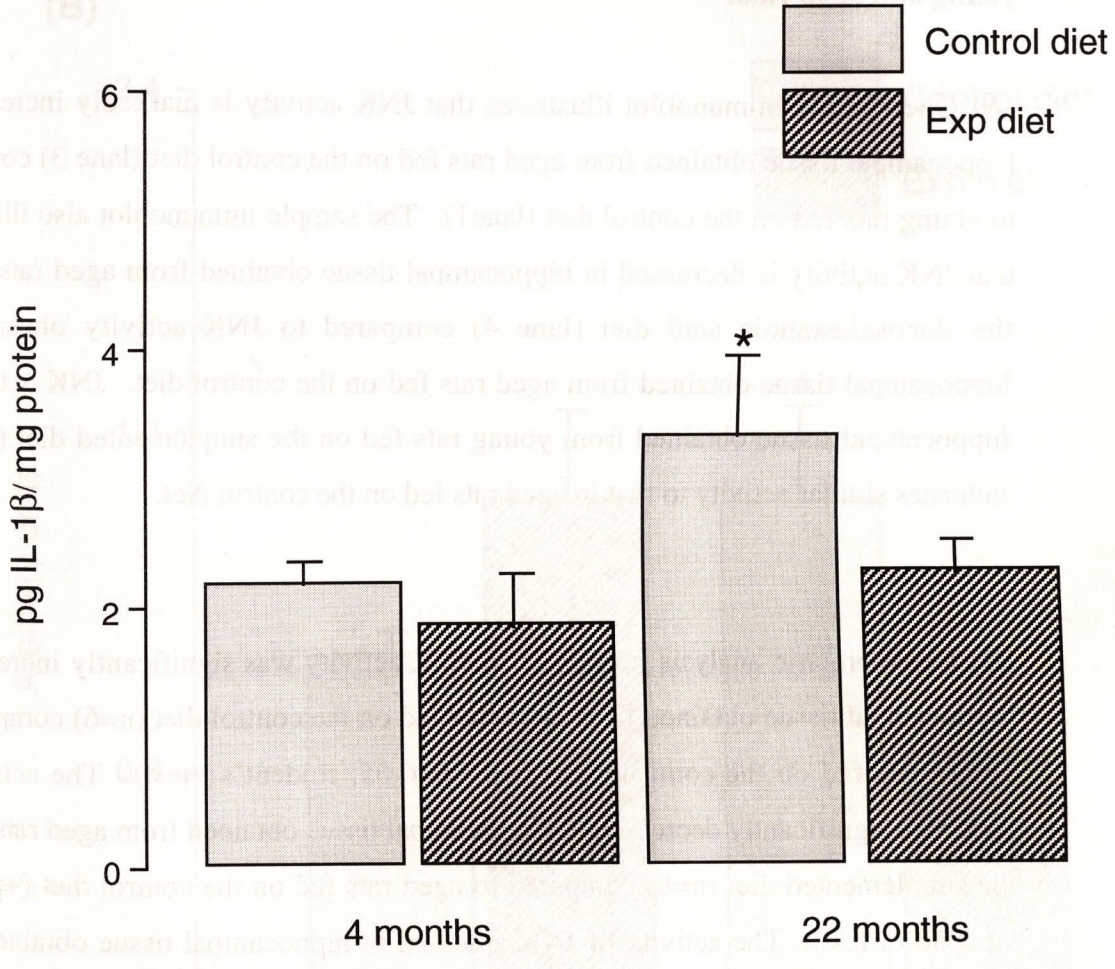
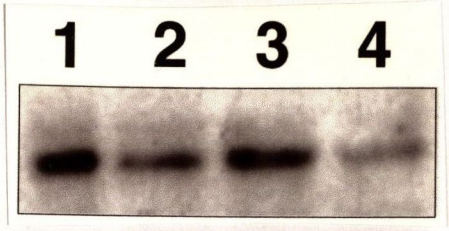

Control diet
Exp diet
pg IL-1β/ mg protein
6
4
2
0
*
4 months
22 months

Fig 5.7 Effect of dietary supplementation on JNK activity in the hippocampus of young and aged rats:

(A) One sample immunoblot illustrates that JNK activity is markedly increased in hippocampal tissue obtained from aged rats fed on the control diet (lane 3) compared to young rats fed on the control diet (lane1). The sample immunoblot also illustrates that JNK activity is decreased in hippocampal tissue obtained from aged rats fed on the docosahexanoic acid diet (lane 4) compared to JNK activity observed in hippocampal tissue obtained from aged rats fed on the control diet. JNK activity in hippocampal tissue obtained from young rats fed on the supplemented diet (lane 2) indicates similar activity to that in aged rats fed on the control diet.

(B) Densitometric analysis revealed that JNK activity was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet (n=6) compared to young rats fed on the control diet (n=3; *$p<0.05$; student's t-test). The activity of JNK was significantly decreased in hippocampal tissue obtained from aged rats fed on the supplemented diet (n=8) compared to aged rats fed on the control diet (+$p<0.05$; student's t-test). The activity of JNK assessed in hippocampal tissue obtained from young rats fed on the supplemented diet (n=3) indicated an increase, compared to young rats fed on the control diet, but this result was not statistically significant. Results are expressed as arbitrary units and are the means ± SEM.

(A)


1 2 3 4

(B)

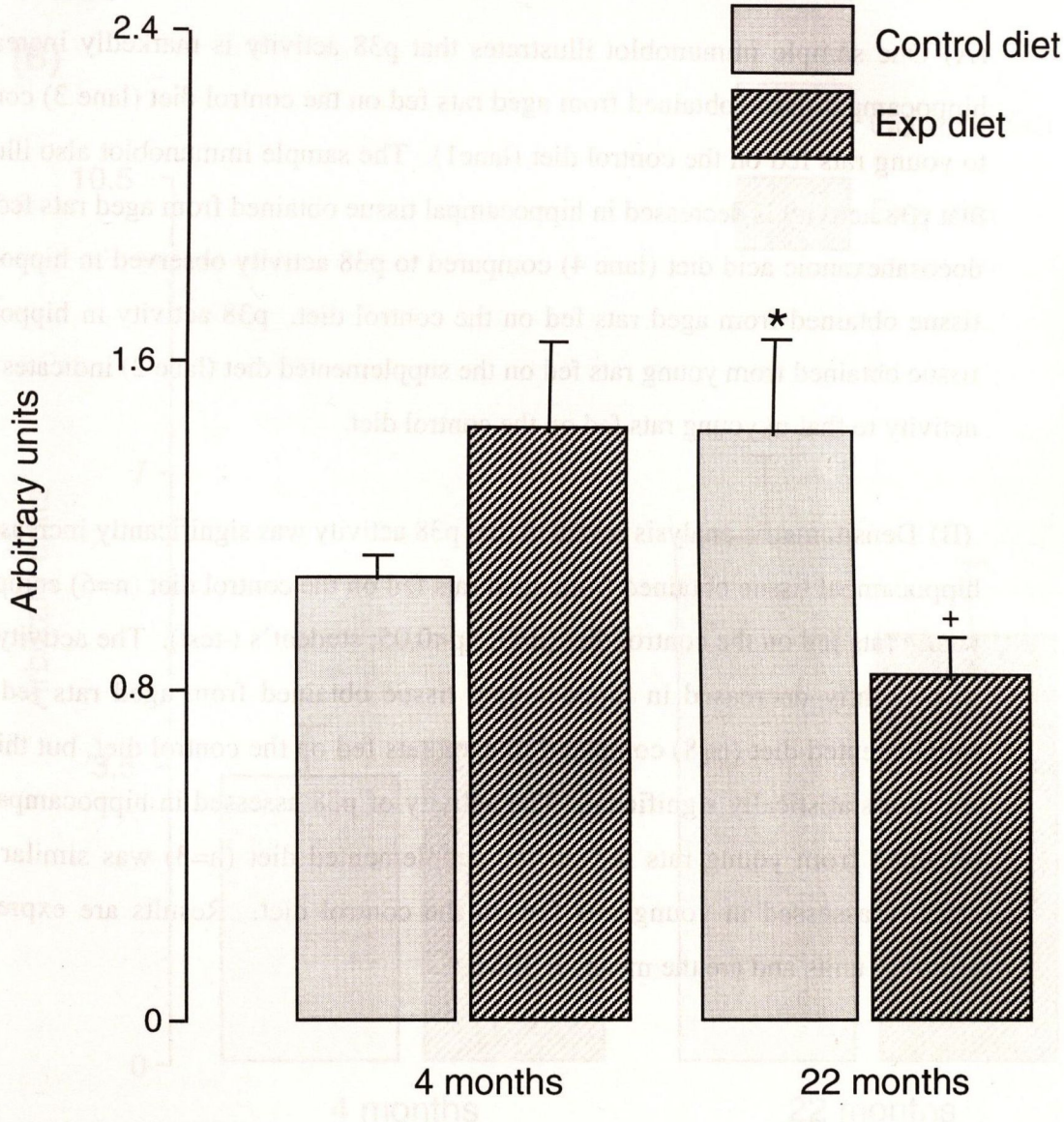
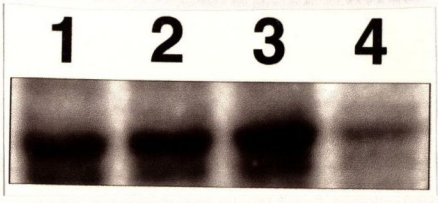

Control diet
Exp diet
2.4
1.6
0.8
0
Arbitrary units
*
+
4 months
22 months

Fig 5.8 Effect of dietary supplementation on p38 activity in the hippocampus of young and aged rats:

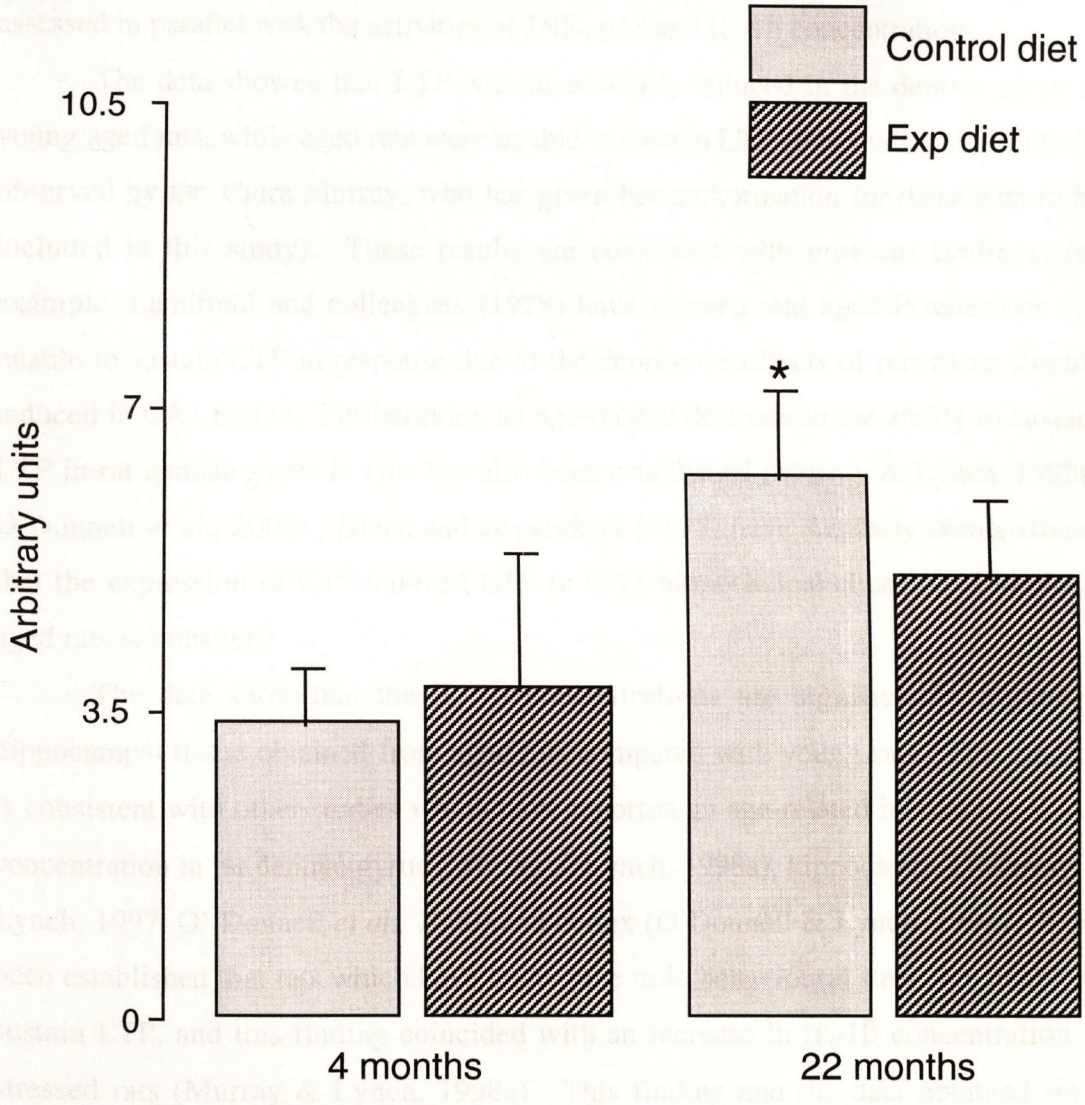
(A) One sample immunoblot illustrates that p38 activity is markedly increased in hippocampal tissue obtained from aged rats fed on the control diet (lane 3) compared to young rats fed on the control diet (lane1). The sample immunoblot also illustrates that p38 activity is decreased in hippocampal tissue obtained from aged rats fed on the docosahexanoic acid diet (lane 4) compared to p38 activity observed in hippocampal tissue obtained from aged rats fed on the control diet. p38 activity in hippocampal tissue obtained from young rats fed on the supplemented diet (lane 2) indicates similar activity to that in young rats fed on the control diet.

(B) Densitometric analysis revealed that p38 activity was significantly increased in hippocampal tissue obtained from aged rats fed on the control diet (n=6) compared to young rats fed on the control diet (n=3; *$p<0.05$; student's t-test). The activity of p38 was slightly decreased in hippocampal tissue obtained from aged rats fed on the supplemented diet (n=8) compared to aged rats fed on the control diet, but this result was not statistically significant. The activity of p38 assessed in hippocampal tissue obtained from young rats fed on the supplemented diet (n=3) was similar to p38 activity assessed in young rats fed on the control diet. Results are expressed in arbitrary units and are the means ± SEM.

(A)


1 2 3 4

(B)


Control diet
Exp diet
10.5
7
3.5
0
Arbitrary units
*
4 months
22 months

5.4 Discussion:

The aims of this study, which were twofold, were prompted by a number of observations reported in the previous chapters. Essentially, these results suggested that induction of LTP was negatively correlated with an increase in IL-1β concentration and the activities of JNK and p38, whereas it appeared that JNK and p38 activity was positively correlated with increased concentrations of IL-1β. The first objective of this study was to establish a role for stress-activated protein kinases in aged rats and assess age-related changes, such as impairment in LTP and increase IL-1β concentration. The second objective was to attempt to reverse any age-related changes by means of dietary supplementation with a polyunsaturated fatty acid, docosahexanoic acid. Subsequent to dietary manipulation, the induction of LTP was assessed in parallel with the activities of JNK, p38 and IL-1β concentration.

The data showed that LTP was successfully induced in the dentate gyrus of young aged rats, while aged rats were unable to sustain LTP (this finding was initially observed by Dr. Ciara Murray, who has given her authorization for these data to be included in this study). These results are consistent with previous findings; for example, Landfield and colleagues (1978) have showed that aged Fischer rats are unable to sustain LTP in response due to the depressive effects of repetitive stimuli induced in CA1 region. Furthermore, an age-related decrease in the ability to sustain LTP in rat dentate gyrus *in vivo* has also been established (Murray & Lynch, 1998a, O'Donnell *et al.,* 2000). Diana and co-workers (1995) have similarly demonstrated that the expression of Ca^{2+}-induced LTP in CA1 hippocampal slices obtained from aged rats is impaired.

The data show that the IL-1β concentrations are significantly higher in hippocampal tissue obtained from aged rats compared with young rats. This finding is consistent with other studies which have reported an age-related increase in IL-1β concentration in rat dentate gyrus (Murray & Lynch, 1998a), hippocampus (Murray & Lynch, 1997, O' Donnell *et al.,* 2000) and cortex (O'Donnell & Lynch, 1998). It has been established that rats which have undergone mild behavioural stress are unable to sustain LTP, and this finding coincided with an increase in IL-1β concentration in stressed rats (Murray & Lynch, 1998a). This finding and the data obtained from previous studies (Murray & Lynch, 1998a,b) together with the current results suggest that one of the underlying causes of impaired LTP observed in aged and stressed rats

is likely to be an elevation in IL-1β. Consistent with the hypothesis that IL-1β concentration is negatively correlated with impaired LTP, increased IL-1β gene expression has been associated with impaired LTP in hippocampal slices and in freely moving rats (Schneider *et al.,* 1998). The proposal that IL-1β exerts an inhibitory effect on LTP is supported by the finding that the IL-1 receptor antagonist (IL-1ra), reversed the IL-1β-induced impairment in LTP (Schneider *et al.,* 1998; Coogan *et al.,* 1999). The inhibitory effect of IL-1β on LTP might be indicative of cytokine-induced deterioration in synaptic function. Such a proposal has been suggested by several previous findings. Enhanced concentrations of IL-1β have been reported in cerebrospinal fluid of Alzheimer's disease and Parkinson's disease patients (Blum-Degen *et al.,* 1995; Malek-Ahmadi, 1998).

The data indicate that activation of both JNK and p38 were increased with age in rat hippocampal tissue. There is a scarcity of literature available on the role of these stress-activated protein kinases in the brain, and their functions in the ageing brain, in particular, have not been elucidated. Nevertheless, one study has shown that JNK activity was enhanced in aged human endothelial cells that underwent shear stress: the enhancement was coupled with elevated blood pressure in humans (Hu *et al.,* 1998). In another study Xiao & Majumdar (2000), investigated the effect of ageing in the gastric mucosa of Fischer rats and reported an increase in the activity of JNK, and that this increase was associated with increased transcriptional activity of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). These observed increases in JNK and relevant transcription factors suggest a role for JNK in age-related increases in gastric mucosal proliferation. However, no change in p38 activity was detected (Xiao & Majumdar, 2000).

The underlying cause of the increase in IL-1β concentration in the aged brain remains to be determined. One proposal is that there is an age-related increase in ROS (O' Donnell *et al.,* 2000) which in turn may stimulate ICE activity and therefore IL-1β concentration. In 1956, Harman presented the free radical theory of ageing, which was based on the assumption that free radicals, which are the products of cellular metabolism have detrimental effects on tissues and that accumulation of these radicals occur in the aged brain (see Nohl, 1993). One of the consequences of increased ROS production is an increase in lipid peroxidation (Rikans & Hornbrook, 1997), and previous studies have shown that associated with an increase in ROS in

aged rats, is enhanced lipid peroxidation and decreased arachidonic acid (AA: Murray & Lynch, 1998a). A causative link has been suggested between the decrease in membrane fluidity, which accompanies a decrease in membrane concentration of polyunsaturated fatty acids such as AA, and impairments in LTP and glutamate release. The proposal that AA plays a significant role in LTP stems from studies undertaken by Williams and colleagues (1989), who showed that infusion of AA induces a form of potentiation that resembles LTP in rat dentate gyrus. Furthermore, it has been shown that AA is released postsynaptically from phospholipids subsequent to the induction of LTP (Clements *et al.,* 1991). A correlation between AA concentration, glutamate release and the ability of aged rats to sustain LTP in rat dentate gyrus *in vivo* has been established (Lynch & Voss, 1994).

To further explore these theories, groups of aged and young rats were fed with a control diet and a diet supplemented with the polyunsaturated fatty acid, docosahexanoic acid. After the period of dietary manipulation, the expression of LTP in the dentate gyrus was assessed and the data represented here indicated that dietary supplementation reversed the age-related impairment in LTP, whereas aged rats fed on the control diet failed to sustain LTP. The docosahexanoic acid diet had no effect on the ability of young rats to sustain LTP. This finding is similar to studies that have shown that dietary manipulation with AA (McGahon *et al.,* 1997), EPA (Martin, *pers. comm.*) and vitamin E and vitamin C (Murray & Lynch, 1998b) reversed the age-related impairment in LTP. Previously it was shown that an age-related decrease in docosahexanoic acid occurs in rat dentate gyrus (McGahon *et al.,* 1999) and that this was associated with an increase in ROS production. These age-related changes were reversed by a diet supplemented with the polyunsaturated fatty acid, docosahexanoic acid (McGahon et al., 1999), and this is consistent with reports suggesting that a decrease in AA is associated with an increase in lipid peroxidation which is a consequence of increased ROS production (Murray & Lynch, 1998a, 1998b).

IL-1β has been proposed as an underlying cause for age-related impairments in LTP and it has been suggested there is a link between IL-1β, lipid peroxidation and ageing. IL-1β has been shown to increase lipid peroxidation in the rat hippocampus and this effect was inhibited by IL-1ra, suggesting that IL-1β may trigger the age-related decrease in AA by increasing ROS and lipid peroxidation (Murray *et al.,* 1997). The data presented here suggest an age-related increase in IL-1β

concentration, which was reversed by the docosahexanoic acid-enriched diet. It could be hypothesized that the diet reversed the age-related increase in IL-1β, therefore lipid peroxidation and ROS formation are downregulated, the effect of which might be coupled with restored AA concentration and membrane fluidity, enabling age rats to sustain LTP. The effect of polyunsaturated fatty acids has been associated with reversed neurological impairments, such as cognitive functions (Kalmijn *et al.*, 1997) and autoimmune diseases (Fernandes *et al.*, 1996). Other reports have examined the correlation between ageing and ROS production through dietary manipulation (Yu *et al.*, 1996).

The data indicate that dietary supplementation reversed the age-related increase in JNK and p38. It could be hypothesized that dietary manipulation exerted this effect because it attenuated the age-related increase in IL-1β, or down-regulated ROS production and these changes were coupled with a decrease in JNK and p38 activity. These data suggest a role for JNK and p38 in age-related changes and it could be hypothesized that they might play a role in mediating the IL-1β-induced increase in lipid peroxidation, thus compromizing the integrity of membrane rigidity, which is associated with an increase in lipid peroxidation (Choe *et al.*, 1995).

The main objective of this study was to assess age-related changes in rat hippocampus and to investigate the effect of dietary supplementation on these changes. The findings outlined in this study are consistent with the hypothesis that aged rats are unable to sustain LTP in perforant path granule cell synapses and this is associated with enhanced concentration of IL-1β and increased activity of JNK and p38 in the rat hippocampus. It could be further hypothesized that these age-related changes are likely to be correlated with enhanced ROS production. Dietary supplementation with docosahexanoic acid reversed the age-related impairment in the ability of rats to sustain LTP, the age-related increase in IL-1β concentration and JNK and p38 activity. These results suggest a role for IL-1β in influencing membrane changes such as increased ROS production, increased lipid peroxidation and decreased AA concentration, whereby all of these factors result in impaired LTP, therefore suggesting their involvement in neurodegeneration. The effect of oxidative stress in Alzheimer's disease has been reviewed by Butterfield and colleagues (1999), where by ROS production appears to be one principal causes in Alzheimer's disease and other neurodegenerative disorders.

Chapter 6

The lipopolysaccharide-induced inhibition of long term-potentiation is coupled with an increase in interleukin-1β converting enzyme/caspase-1

6.1 Introduction:

Interleukin-1β (IL-1β) has been implicated in the modulation of brain functions such as systemic host responses to disease and local changes caused by peripheral inflammation, infection and insult (Rothwell, 1999). IL-1β is also thought to be involved in the pathology of chronic infectious diseases and is essential in the induction of the acute-phase response, a comprehensive part of our ability to fight off infections caused by intracellular and extracellular bacteria (Henderson *et al.,* 1996). IL-1β can be stimulated by various agents, such as lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria such as *Bordetella Pert*usis, *E. Coli* and *Helicobacter Pylori.* Since LPS has the ability to stimulate cytokine synthesis and modulate cellular activity, it has been widely used to mimic the acute-phase response that results in cytokine synthesis (Mohankumar *et al.,* 1999; Nick *et al.,* 2000). LPS has been shown to up-regulate the expression of IL-1β protein and IL-1β mRNA in specific brain regions following its intracerebroventricular administration (Ilyin *et al.,* 1998). *In vitro* experiments have also shown an LPS-induced increase in IL-1β concentration in the mouse hippocampus (Loscher, *pers comm.*). Similarly, peripheral administration of LPS increases the expression of IL-1β at the mRNA and protein levels in the spleen, pituitary and brain of mice (Goujon *et al.,* 1996).

IL-1β is one of the primary pro-inflammatory mediators in the pathogenesis of both chronic and acute inflammation (Thornberry *et al.,* 1992; Ayala *et al.,* 1994) and is synthesized in immune cells such as macrophages and also in neuronal and glial cells (Besedovsky *et al.,* 1998). In order for IL-1β to be activated, it must be converted from the inactive pro-IL-1β to an active form, which is achieved by a cytoplasmic cysteine protease known as IL-1β-converting enzyme (ICE; Thornberry *et al.*, 1992). The predominant form of ICE is p45, which has the ability to cleave pro-IL-1β only after it is degraded to the p20/p10 isoforms (Miller *et al.*, 1994). Although IL-1β has been proposed to be neuroprotective in certain circumstances, it is generally believed that prolonged exposure of IL-1β can result in neurodegenerative effects (Rothwell, 1999). An outstanding example of neuronal

deficit induced by IL-1β is the impairment in long-term potentiation (LTP) in the CA1 rat hippocampal slices *in vitro* (Bellinger *et al.,* 1993), CA3 hippocampal slices (Katsuki *et al.,* 1990) and in rat dentate gyrus *in vivo* (Murray & Lynch, 1998a). It may be hypothesized that IL-1β-associated changes, such as those induced by LPS, may occur by an increase in the activity of ICE. Hara and colleagues (1997) have shown that ICE inhibitors decreased neuronal deficit in ischemic mouse brain and reduced tissue damage in the rat brain, supporting the hypothesis that ICE plays a pivotal role in LPS-induced neuronal changes.

LPS has been shown to activate the stress-activated protein kinases, JNK and p38, in many inflammatory cells such as macrophages, neutrophils and endothelial cells (Pyo *et al.*, 1998). However activation of specific kinases depends on the cell type. For instance, LPS causes activation of all members of the MAPK superfamily in macrophages, but in neutrophils only p38 is activated (Pyo *et al.*, 1998). Similarly, LPS-treated cultured rat microglia and astrocytes, show increased activation of p38 (Bhat *et al.,* 1998). LPS-induced JNK activation has been reported in various cell types, such as neutrophils (Nick *et al.,* 1996) and macrophages (Nick *et al.,* 1996, Hambleton *et al.,* 1996, Mackichan & DeFranco, 1999).

The aim of this study was to examine the effect of intraperitoneal administration of LPS on LTP, IL-1β concentration and glutamate release in the rat dentate gyrus. The substrates for modulation by LPS that were assessed in this study were JNK, p38 and reactive oxygen species formation. Since LPS has been shown to induce IL-1β in neutrophils, macrophages and cultured rat microglia, we tested the hypothesis that the modulatory effects of LPS on LTP arise from an LPS-induced increase in the expression of IL-1β in the hippocampus. To do so, animals were treated intracerebroventricularly (i.c.v.) with the ICE inhibitor peptide Ac-YVAD-CMK or saline and injected i.p. with either LPS or saline. The effect of the ICE inhibitor peptide on the LPS-associated changes in expression of LTP were assessed in conjunction with IL-1β concentration, ICE and JNK activity.

6.2 Methods

6.2.1 Treatment regimes

Six groups of male Wistar rats were anaesthetized intraperitoneally (i.p.) with urethane (1.5g/kg). The head was positioned in a head holder within a sterotaxic frame. All groups of rats received saline (1ml: 0.9%) or LPS (1ml: 100µg/kg) i.p.; four groups were pretreated either with an intracerebroventricular (i.c.v.) injection of saline (5µl: 0.9%) or the ICE inhibitor, Ac YVAD-CMK (5µl: 10pmol) 3 h prior to induction of LTP. I.c.v. injections were administered as described (section 2.5.4) and the rats were monitored for this subsequent 3 h period. This treatment protocol is illustrated in table 1.

Table 1. **Treatment regimes**

I.C.V. (n=6)	**I.P.** (n=6)
	Saline (1ml: 0.9%)
	LPS (1ml: 100µg/kg)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

6.2.2 Induction of LTP *in vivo*

3 h following treatment, rats were positioned in a sterotaxic frame and stimulating and recording electrodes were positioned (see section 2.5.2 and 2.5.3) and recordings at test shock frequency were made for 10 min prior, and 40 min after, tetanic stimulation (3 trains of stimuli; 250 Hz for 200 msec).

6.2.3 Tissue storage

At the end of the electrophysiological recording period, rats were killed by cervical dislocation as described (section 2.3). The hippocampus was removed and the untetanized and tetanized dentate gyri, as well as hippocampus proper, were dissected on ice and prepared for storage as described (see section 2.3.1).

6.2.4. Analysis of glutamate release

Impure synaptosomal preparations, P_2, were prepared from untetanized and tetanized dentate gyrus as described (see section 2.3.2). The synaptosomal pellet was resuspended in Krebs solution containing $CaCl_2$ (final concentration: 2mM), synaptosomes were aliquotted onto a Millipore filtration manifold, washed under vacuum, and release was assessed in the absence or presence of 50mM KCl. Filtrates were collected and analysed by immunoassay (see section 2.7) and results were expressed as µmol glutamate/mg protein.

6.2.5 Analysis of JNK and p38 activity

The activities of JNK and p38 were assessed in P_2 obtained from the hippocampus of saline- and LPS-treated groups. The activity of JNK was only assessed in the four groups pretreated either with saline and LPS (i.p.) and saline and the ICE inhibitor (i.c.v.). The analysis of JNK and p38 was assessed as described in section 2.8.1 and 2.8.2. Briefly, proteins were separated by gel electrophoresis, transferred onto nitrocellulose paper and immunoblotted with anti-phospho JNK or anti-phospho p38 antibodies (section 2.8.3). The antibody complex was visualized using ECL detection and protein bands were quantified by densitometric analysis and results were expressed as arbitrary units.

6.2.6 Analysis of IL-1β concentration

The concentration of IL-1β was assessed in hippocampal homogenate or untetanized or tetanized dentate gyrus obtained from pretreated rats (section 2.6.1). Slices were homogenized in lysis buffer (500µl; 25mM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml pepstatin) and protein concentration was equalized (section 2.4). Briefly, a 96-well microtiter plate was coated with capture and standards (range: 0-1000pg/ml) or samples were incubated for 1 h prior to the addition of the secondary antibody. The detection antibody reagent and the working substrate, TMB, were added, the reaction was stopped by the addition of 2N H_2S0_4 and the samples were read at 450nm (section 2.9). IL-1β concentration was expressed as pg IL-1β/mg protein.

6.2.7 Analysis of Interleukin1β Converting Enzyme (ICE) activity

Slices of hippocampus were homogenised in lysis buffer (400µl; 25mM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml leupeptin, 10µg/ml pepstatin), put through 4 freeze thaw cycles and centrifuged for 20 min at 15,000 rpm at 4°C. Supernatant was removed (90µl), added to 500µM ICE substrate (10µl: DEVD peptide) and incubated for 1h at 37°C as previously described (see section 2.10), transferred to cuvettes and analysed by fluorometry. Results were expressed as nmol AFC/mg protein/min.

6.2.8 Analysis of reactive-oxygen species (ROS) formation

ROS formation was assessed in hippocampal synaptosomes prepared from saline- and LPS-treated tissue using the non-fluorescent probe 2'7'-dichlorofluorescein diacetate (DCFH-DA). Synaptosomes were resuspended in 1 ml of ice-cold Tris buffer (40mM; pH 7.8), incubated in the presence of the DCFH-DA probe (10µl; final concentration 5µM) at 37°C for 15 min and then centrifuged at 13,000 rpm for 8 min. The pellet was resuspended in Tris buffer (40mM; pH 7.8), and fluorescence was monitored. Results were expressed as nmol DCF/mg protein

6.3 Results

6.3.1 Effect of LPS treatment on LTP

Analysis of data indicates that i.p. injection of LPS inhibited LTP in perforant path cell synapses (fig. 6.1). Tetanic stimulation delivered to the perforant path 3 h after i.p. injection of LPS resulted in an increase in the mean epsp slope, the mean percentage increase in the 2 min immediately following tetanic stimulation (± SEM; compared with the 5 min immediately prior to tetanic stimulation) was 133.58 ± 3.48 % (SEM; n=6) but was this was not maintained so that the mean epsp slope in the last 5 min of the experiment was 100.81 ± 2.26 %. The corresponding values in the saline-treated control rats were 164.83 ± 4.23 % and 119.1 ± 2.17 %, respectively.

6.3.2 Effect of LPS on endogenous glutamate release

Glutamate release was assessed in synaptosomes prepared from untetanized and tetanized dentate gyrus obtained from saline and LPS pretreated rats. Fig 6.2 indicates that the addition of 50mM KCl significantly increased glutamate release in synaptosomes prepared from untetanized dentate gyrus of saline-treated rats (*$p<0.05$; student's t-test). The mean values were 0.61 ± 0.2 μmol/mg (SEM; n=6) and 1.36 ± 0.33 μmol/mg, for unstimulated and KCl-stimulated glutamate release, respectively. The addition of 50mM KCl caused a further significant increase in glutamate release in synaptosomes prepared from tetanized dentate gyrus of saline-treated rats (**$p<0.01$; student's t-test). The mean values were 0.76 ± 0.34 μmol/mg (SEM; n=6) and 1.97 ± 0.44 μmol/mg for unstimulated and KCl-stimulated glutamate release, respectively. In LPS-treated rats, KCl failed to stimulate glutamate release in synaptosomes prepared from untetanized dentate gyrus. The mean values were 0.72 ± 0.26 μmol/mg and 0.61 ± 0.33 μmol/mg for unstimulated and KCl-stimulated release, respectively. Similarly, KCl failed to stimulate glutamate release in synaptosomes prepared from tetanized dentate gyrus obtained from LPS-treated rats. The mean values were 0.45 ± 0.16 μmol/mg and 0.84 ± 0.12 μmol/mg for unstimulated and KCl-stimluated release, respectively. KCl-stimulated glutamate release was significantly decreased in tissue prepared from both untetanized and tetanized dentate gyrus synaptosomes prepared from LPS-treated, compared with saline-treated rats (+$p<0.05$; student's t-test).

6.3.3 Eeffect of LPS injection on IL-1β concentration

IL-1β concentration was assessed in synaptosomes obtained from untetanized and tetanized dentate gyrus of saline- and LPS-treated rats. The data illustrated in fig. 6.3 indicate that IL-1β concentration was significantly increased in synaptosomes obtained from untetanized dentate gyrus of LPS-treated rats compared to synaptosomes obained from untetanized and tetanized dentate gyrus of saline-treated rats (*$p<0.05$; student's t-test). The mean values were 103.9 ± 40.1 pg/mg (SEM; n=5) and 102.99 ± 19.42 pg/mg for synaptosomes obtained from tetanized and untetanized dentate gyrus of saline-treated rats respectively and 377.9 ± 104.6 pg/mg

(n=6) for synaptosomes prepared from untetanized dentate gyrus of LPS-treated rats. IL-1β concentration assessed in synaptosomes prepared from tetanized dentate gyrus of LPS-treated rats was similar to synaptosomes prepared from untetanized dentate gyrus of LPS-treated rats, where the mean values was 302.22 ± 124.17 pg/mg, but this result was not statistically significant. The data also indicate that teteanic stimulation did not effect IL-1β concentration in tissue prepared from saline- and LPS-treated rats.

6.3.4 Effect of LPS on ICE activity

The data indicate that ICE activity was significantly increased in hippocampal tissue prepared from LPS-treated rats compared with saline-treated rats (*$p<0.05$; student's t-test; fig. 6.4). The mean values were 2.79 ± 0.38 nmol/mg/min (SEM: n=6) and 7.91 ± 1.7 nmol/mg/min (SEM: n=5) for saline- and LPS-treated rats, respectively.

6.3.5 Effect of LPS on JNK activity:

Activity of JNK was assessed in hippocampal tissue obtained from saline- and LPS-treated rats. The sample immunoblot illustrated in fig. 6.5 (A) illustrates that JNK activity was increased in hippocampal tissue obtained from LPS-treated rats compared with saline-treated rats. The mean data, obtained from densitometric analysis, are shown in fig. 6.5 (B). The mean values, expressed in arbitrary units show that JNK activity was significantly increased from 7.59 ± 0.71 (SEM; n=6) to 11.74 ± 1.1 in saline- and LPS-treated rats, respectively

6.3.6 Effect of LPS on p38 activity:

Activity of p38 was assessed in hippocampal tissue obtained from saline- and LPS-treated rats. The sample immunoblot illustrated in fig. 6.6 (A) illustrates that p38 activity was increased in hippocampal tissue obtained from LPS-treated rats compared with saline-treated rats. The mean data, obtained from densitometric analysis, are shown in fig. 6.6 (B). The mean values, expressed in arbitrary units show that p38 activity was significantly increased from 2.94 ± 0.84 (SEM; n=6) to 5.28 ± 0.8 in saline- and LPS-treated rats, respectively.

6.3.7 Effect of LPS on reactive oxygen species (ROS) production

ROS production was assessed in hippocampal synaptosomes prepared from saline- and LPS-treated rats. Fig. 6.7 shows that ROS production was significantly increased in hippocampal synaptosomes prepared from LPS-treated rats compared with synaptosomes prepared from saline-treated rats (*$p<0.05$; student's t-test). The mean values were 4.75 ± 0.6 pmol/mg (SEM: n=6) and 6.95 ± 0.59 pmol/mg in tissue prepared from saline- and LPS-treated rats, respectively.

6.3.8 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced inhibition of LTP

Fig 6.8 indicates that while LPS inhibited the expression of LTP the LPS-induced inhibition of LTP was blocked by the ICE inhibitor. The mean percentage change in epsp slope in the 2 min immediately following tetanic stimulation was 177.77 ± 15.34 % (SEM: n=6) in the control group (treated with saline i.c.v and i.p.) compared with 118.92 ± 3.35 % in the group treated with saline (i.c.v.) and LPS (i.p.). In the last 5 min of the experiment the values were 123.86 ± 2.14 % and 96.21 ± 1.14 % respectively. Injection of the ICE inhibitor partially reversed the LPS-induced inhibition of LTP in the earlier phase induced by tetanic stimulation and completely blocked the LPS-induced inhibition of the later phase of LTP. The mean percentage changes were 147.32 ± 9.23 % and 123.54 ± 6.33 % in the first 2 min after tetanic stimulation and in the last 5 min of the experiment respectively. However the expression of LTP was similar in control rats and the group injected with the ICE inhibitor (i.c.v.) saline (i.p.). In the latter group the mean percentage changes in epsp slope were 174.57 ± 16.24 % and 142.07 ± 7.42 % in the first 2 min after tetanic stimulation and in the last 5 min of the experiment respectively.

6.3.9 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in IL-1β concentration

IL-1β concentration was assessed in hippocampal tissue prepared from saline- and LPS-treated rats that were injected intracerebroventricularly (i.c.v.) with either saline or the ICE inhibitor. The data illustrated in fig. 6.9 indicates that IL-1β concentrations were significantly increased in hippocampal tissue prepared from LPS

pretreated rats, injected (i.c.v.) with saline compared with rats which only received saline (*p<0.0.5; student's t-test). The mean values were 132.36 ± 12.9 pg/mg (SEM: n=8) and 196.83 ± 26.9 pg/mg for saline- and LPS-treated rats, where both groups were injected (i.c.v.) with saline. The data also revealed that the LPS-induced increase in IL-1β concentration was attenuated in hippocampal tissue obtained from LPS-treated rats injected with the ICE inhibitor (+p<0.05; student's t-test); the mean value was 121.74 ± 13.81 pg/mg. IL-1β concentration assessed in hippocampal tissue obtained from saline-treated rats injected with the ICE inhibitor was similar to that of the control group, where the mean value was 135.66 ± 26.92 pg/mg.

6.3.10 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in ICE activity

ICE activity was assessed in hippocampal tissue prepared from rats pre-treated with saline and LPS and injected intracerebroventricularly with either saline or the ICE inhibitor. The data illustrated in fig. 6.10 indicates that ICE activity was significantly increased in LPS pretreated rats, injected i.c.v. with saline compared with rats treated with saline alone (*p<0.0.5; student's t-test). The mean values were 2.14 ± 0.5 nmol/mg/min (SEM: n=13) and 4.9 ± 1.07 nmol/mg/min (SEM: n=14) for saline- and LPS-treated rats respectively, where both groups were injected i.c.v. with saline. The data also revealed that the LPS-induced increase in ICE activity was significantly attenuated in hippocampal tissue obtained from LPS-treated rats injected i.c.v. with the ICE inhibitor (+++p<0.001; student's t-test); the mean value was 0.617 ± 0.25 nmol/mg/min (n=8). ICE activity assessed in hippocampal tissue obtained from saline-treated rats injected i.c.v. with the ICE inhibitor was similar to that of the control group, where the mean value was 0.89 ± 0.35 nmol/mg/min (n=7).

6.3.11 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in JNK activity

JNK activity was assessed in hippocampal tissue prepared from rats treated with saline or LPS and pre-injected i.c.v. with either saline or the ICE inhibitor. The sample immunoblot shown in fig. 6.11 (A) illustrates that the LPS-induced increase in JNK activity is attenuated with the ICE inhibitor. The mean data (fig. 6.11) obtained

from densitometric analysis and expressed in arbitrary units indicates that JNK activity was significantly increased in tissue prepared from LPS treated rats, compared with rats treated with saline alone (*p<0.0.5; student's t-test). The mean values were 65.01 ± 0.48 (SEM: n=3) and 122.29 ± 18.36 for saline- and LPS-treated rats respectively, where both groups were injected i.c.v. with saline. The LPS-induced increase in JNK activity was markedly attenuated in hippocampal tissue obtained from LPS-treated rats injected i.c.v. with the ICE inhibitor. The mean value was 80.9 ± 13.9 while JNK activity assessed in hippocampal tissue obtained from saline-treated rats injected (i.c.v.) with the ICE inhibitor was 85.48 ± 0.10.

Fig. 6.1 Effect of LPS on LTP:

Male Wistar rats were anaesthetized by intraperitoneal (i.p.) injection of urethane (1.5g/kg). The rats were injected i.p. with either LPS (1ml: 100μg/kg) or saline (1ml: 0.9%) 3 hrs prior to LTP. Test shocks were given at 30 sec intervals for a 10 min control period prior to tetanization. High frequency stimulation, consisting of 3 trains of stimuli of 250 Hz for 200 msec at 30 sec intervals, was delivered to the perforant path, as indicated by the arrow. LTP was induced and maintained in saline-treated rats, whereas LPS-treated rats failed to sustain LTP. The scale bars represent 1 mV and 2 msec. SEM values are included for every tenth response. Mean epsp slope for each group is expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation and the data is presented as the mean change with time. Results are means ± SEM of 6 independent observations for both saline and LPS treated rats.

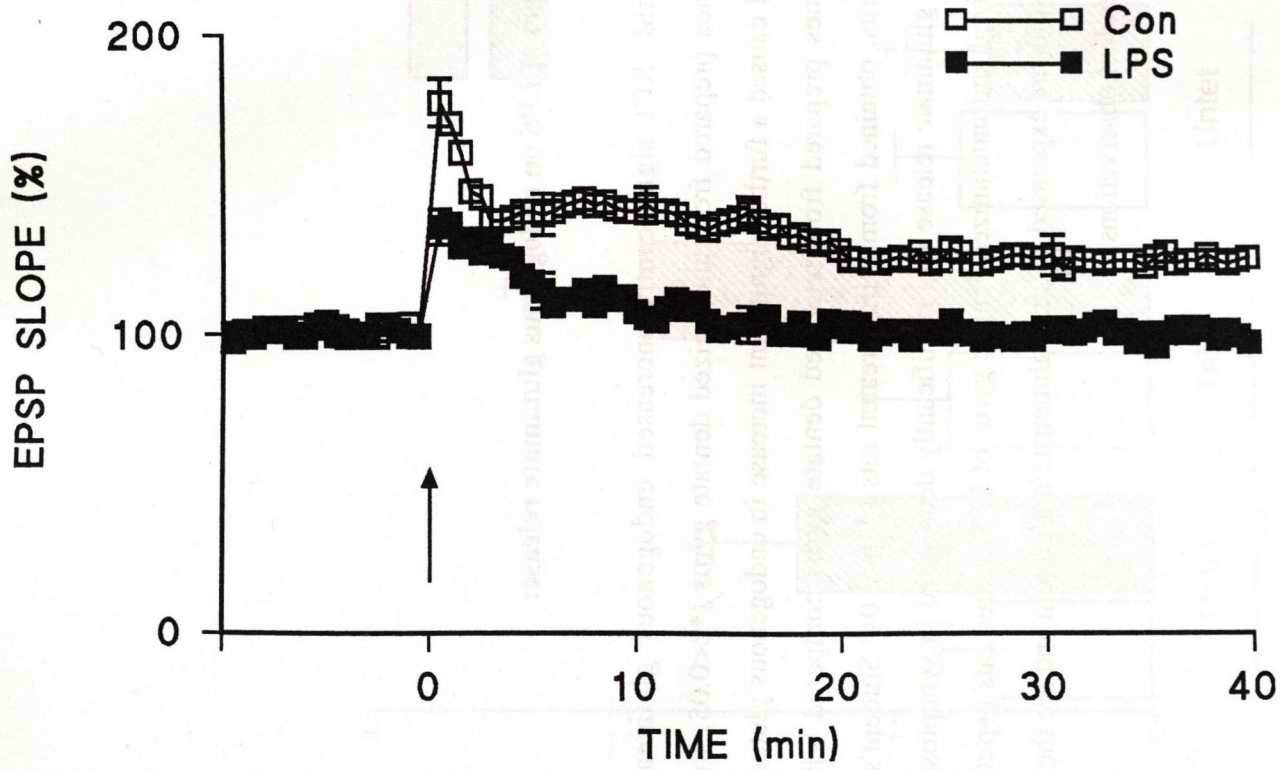

Con
LPS
200
100
0
EPSP SLOPE (%)
0
10
20
30
40
TIME (min)

Fig 6.2 Effect of LPS on endogenous glutamate release:

50mM KCl significantly increased endogenous glutamate release in synaptosomes prepared from untetanized dentate gyrus (*$p<0.05$; student's t-test). 50mM KCl caused a further significant increase in endogenous glutamate release in synaptosomes prepared from tetanized dentate gyrus compared with untetanized dentate gyrus, obtained from saline-treated rats (**$p<0.01$; Student's t-test). KCl-stimulated glutamate release was significantly decresased in synaptosomes prepared from tetanized and untetanized dentate gyrus of LPS-treated rats (+$p<0.05$; Student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 6 independent observations.

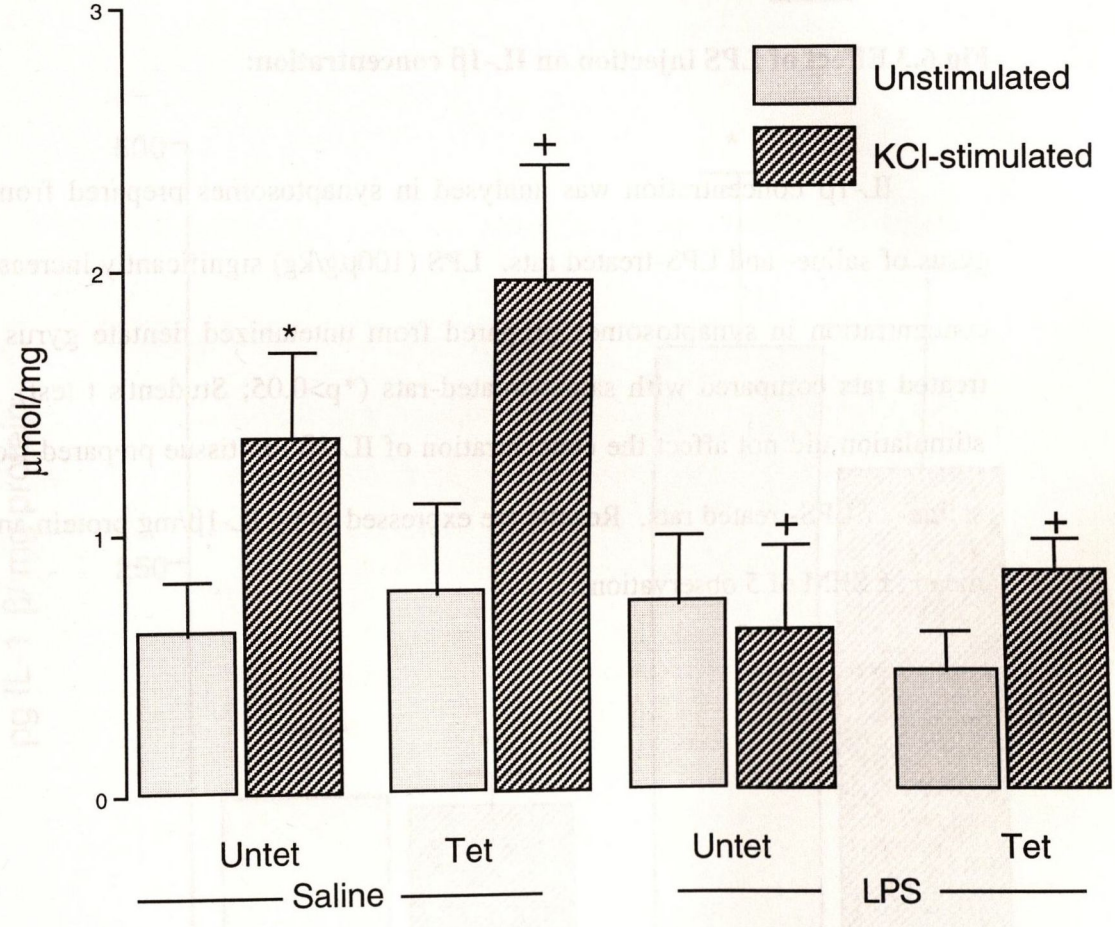

Unstimulated
KCl-stimulated
µmol/mg
3
2
1
0
*
+
+
+
Untet
Tet
Untet
Tet
Saline
LPS

Fig 6.3 Effect of LPS injection on IL-1β concentration:

IL-1β concentration was analysed in synaptosomes prepared from dentate gyrus of saline- and LPS-treated rats. LPS (100μg/kg) significantly increased IL-1β concentration in synaptosomes prepared from untetanized dentate gyrus of LPS-treated rats compared with saline-treated-rats (*$p>0.05$; Student's t-test). Tetanic stimulation did not affect the concentration of IL-1β ion tissue prepared from either saline- or LPS-treated rats. Results are expressed as pg IL-1β/mg protein and are the means ± SEM of 5 observations.

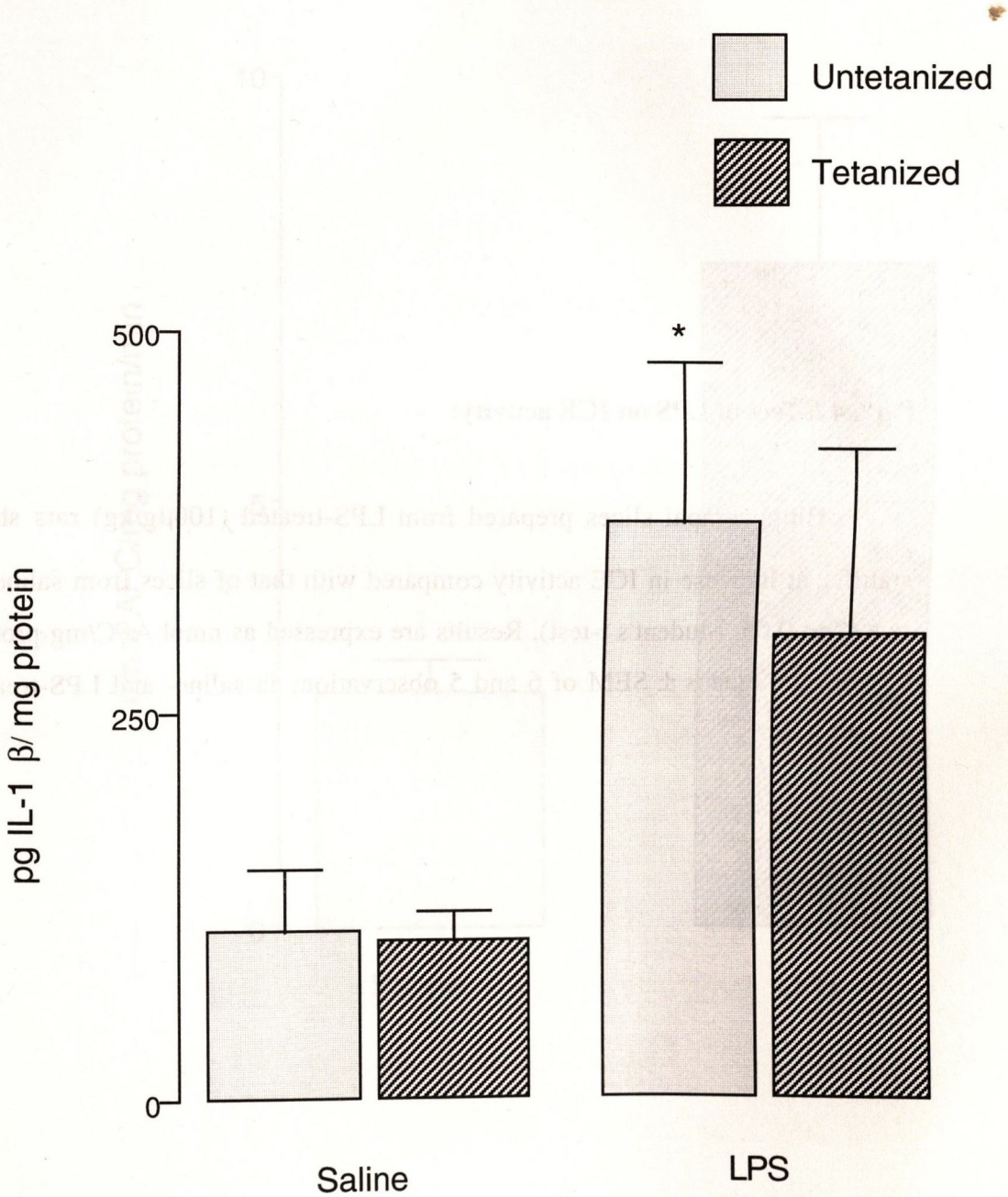

Untetanized
Tetanized
500
250
0
pg IL-1 β/ mg protein
*
Saline
LPS

Fig 6.4 Effect of LPS on ICE activity:

Hippocampal slices prepared from LPS-treated (100μg/kg) rats showed a significant increase in ICE activity compared with that of slices from saline-treated rats (*$p < 0.05$; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are the means ± SEM of 6 and 5 observations in saline- and LPS-treated rats respectively.

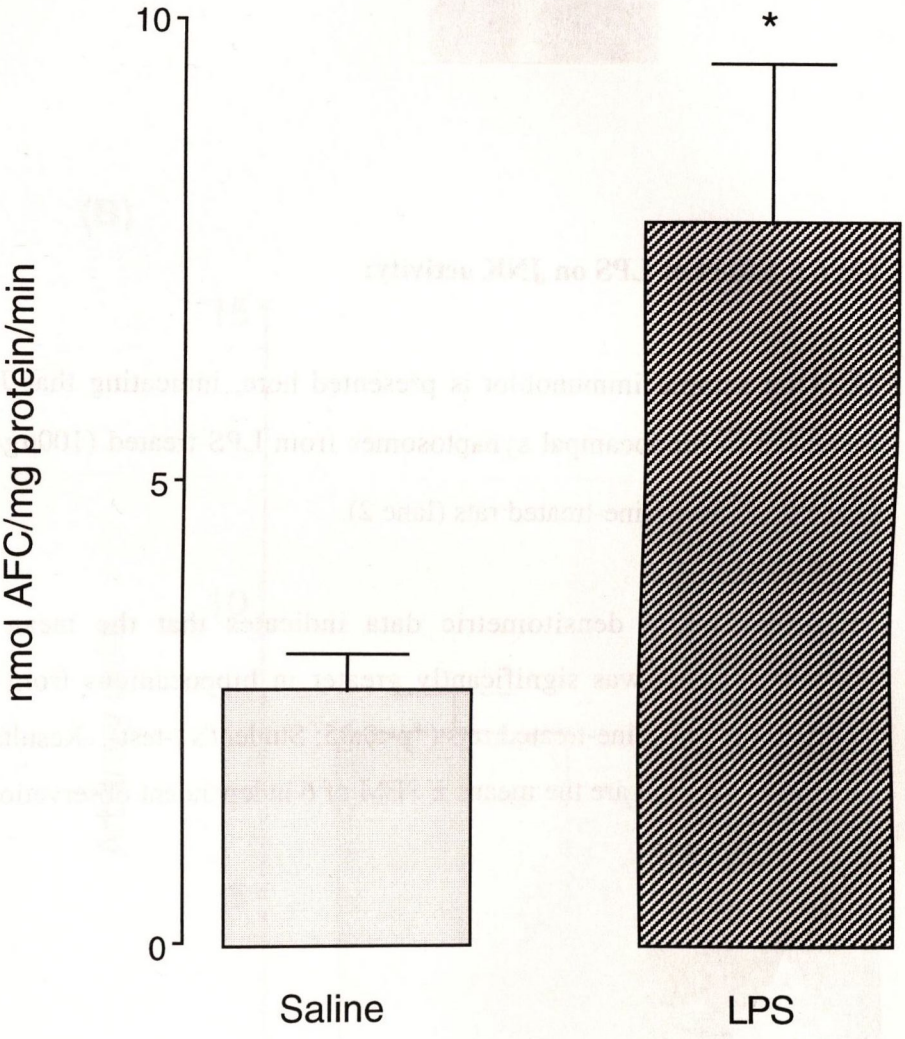
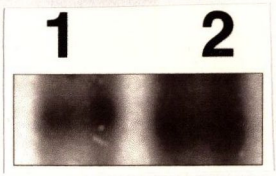

10
5
0
nmol AFC/mg protein/min
*
Saline
LPS

Fig 6.5 Effect of LPS on JNK activity:

(A) One sample immunoblot is presented here, indicating that JNK activity was increased in hippocampal synaptosomes from LPS-treated (100μg/kg) rats (lane 1) compared with saline-treated rats (lane 2).

(B) Analysis of densitometric data indicates that the mean value for JNK phosphorylation was significantly greater in hippocampus from LPS-treated rats compared with saline-treated rats (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are the means ± SEM of 6 independent observations.

(A)



(B)

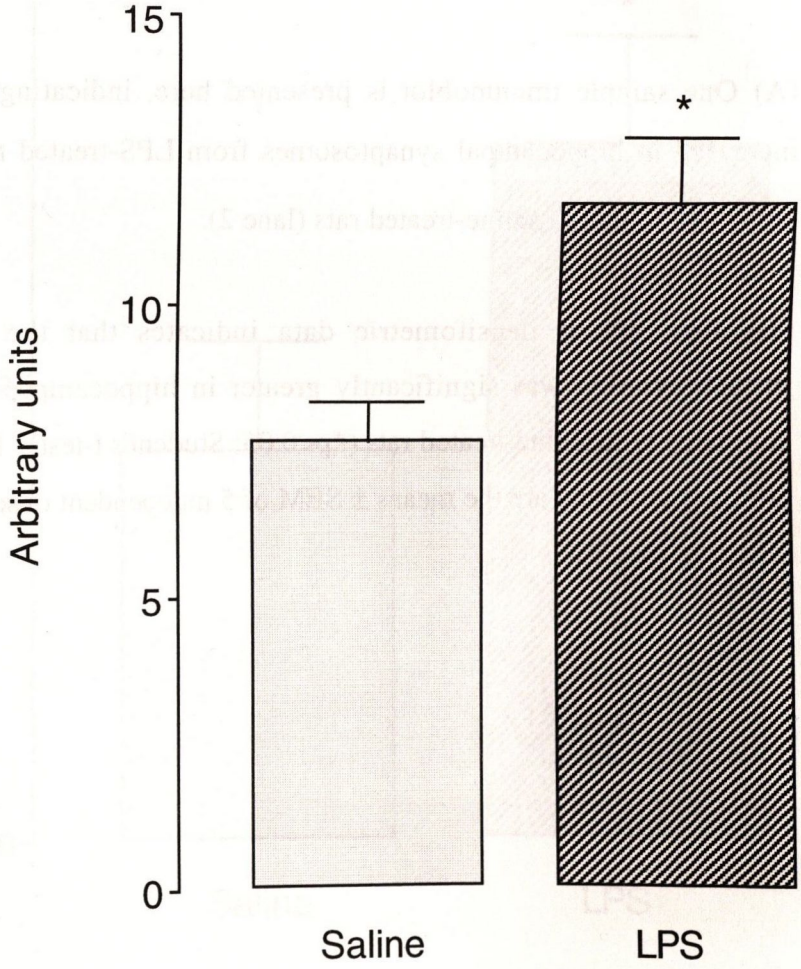


Fig. 6.6 Effect of LPS on p38 activity:

(A) One sample immunoblot is presented here, indicating that p38 activity was increased in hippocampal synaptosomes from LPS-treated rats (100μg/kg; lane 1) compared to that of saline-treated rats (lane 2).

(B) Analysis of densitometric data indicates that the mean value for p38 phosphorylation was significantly greater in hippocampus from LPS-treated rats compared with saline-treated rats (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are the means ± SEM of 5 independent observations.

(A)

(B)

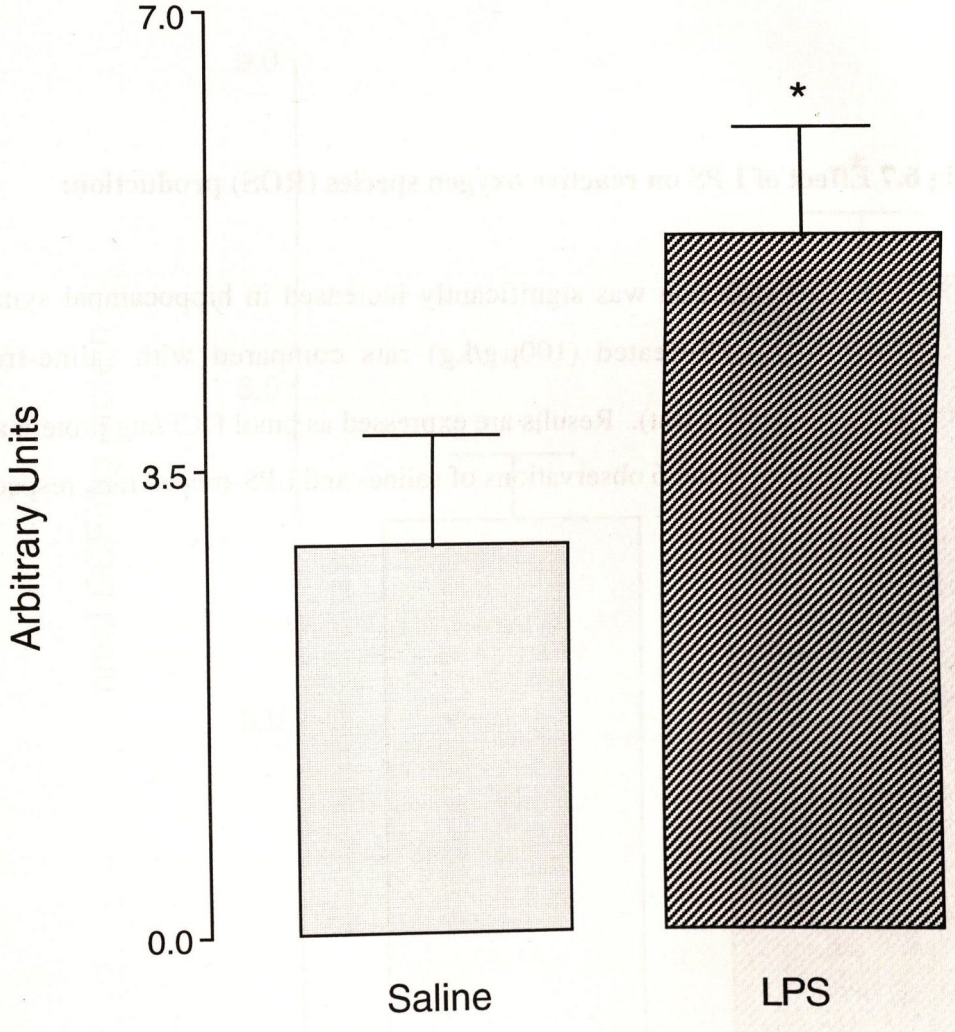


Fig 6.7 Effect of LPS on reactive oxygen species (ROS) production:

ROS production was significantly increased in hippocampal synaptosomes prepared from LPS-treated (100μg/kg) rats compared with saline-treated rats (*$p<0.05$; Student's t-test). Results are expressed as pmol DCF/mg protein and are the means ± SEM of 4 and 6 observations of saline- and LPS-treated rats, respectively.

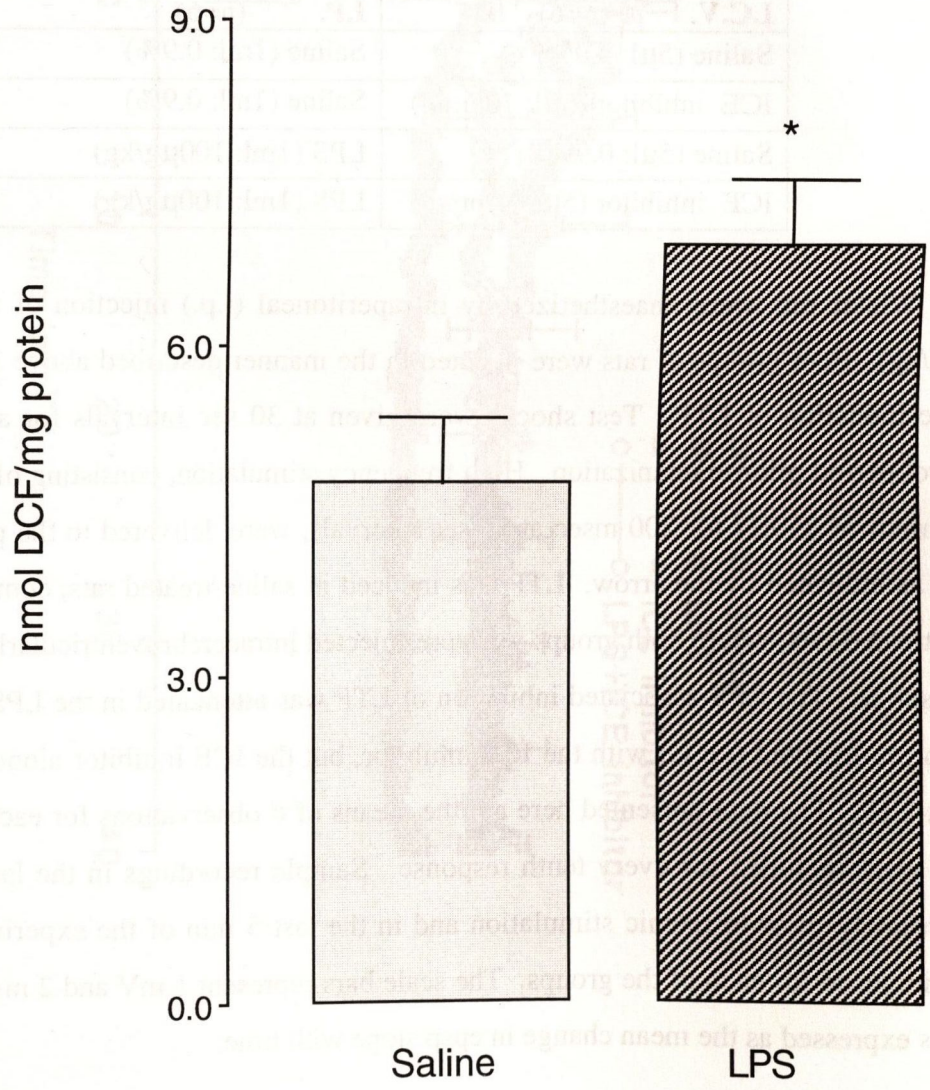

9.0
6.0
3.0
0.0
nmol DCF/mg protein
*
Saline
LPS

Fig 6.8 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced inhibition of LTP

The expression of LTP was assessed in rat dentate gyrus in 4 groups of rats treated as follows:

Treatment regimes

I.C.V. (n=6)	**I.P.** (n=6)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

Male Wistar rats were anaesthetized by intraperitoneal (i.p.) injection of urethane (1.5g/kg). Four groups of rats were injected in the manner described above 3 h prior to the induction of LTP. Test shocks were given at 30 sec intervals for a 10 min control period prior to tetanization. High frequency stimulation, consisting of 3 trains of stimuli of 250Hz for 200 msec at 30 sec intervals, were delivered to the perforant path, as indicated by the arrow. LTP was induced in saline-treated rats, compared to LPS-treated rats, where both groups were pre-injected intracerebroventricularly (i.c.v.) with saline. The LPS-associated inhibition of LTP was attenuated in the LPS-treated group pre-injected (i.c.v.) with the ICE inhibitor, but the ICE inhibitor alone did not affect LTP. The data presented here are the means of 6 observations for each group; SEM were included for every tenth response. Sample recordings in the last 5 min immediately prior to tetanic stimulation and in the last 5 min of the experiment are superimposed for each of the groups. The scale bars represent 1 mV and 2 msec. The data is expressed as the mean change in epsp slope with time.

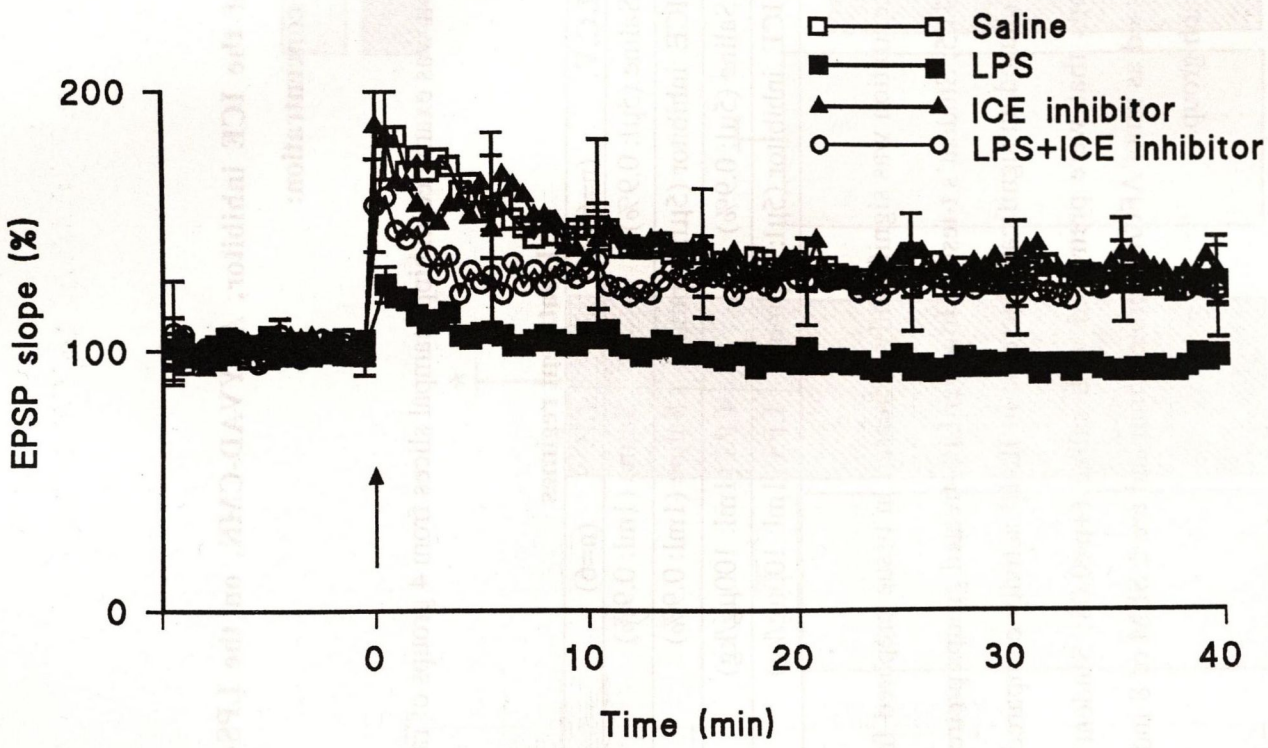

Saline
LPS
ICE inhibitor
LPS+ICE inhibitor
200
100
0
EPSP slope (%)
0
10
20
30
40
Time (min)

Fig 6.9 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in IL-1β concentration:

IL-1β concentration was examined in hippocampal slices from 4 groups of rats treated as follows:

Treatment regimes

I.C.V. (n=6)	**I.P.** (n=6)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

Mean IL-1β concentration was significantly increased in tissue prepared from LPS-treated rats. (*$p<0.05$; Student's t-test). However LPS-treated groups pretreated with the ICE inhibitor caused an significant decrease in IL-1β activity compared with the LPS-treated groups that were pretreated with saline (+$p<0.05$; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are ± SEM of 8 independent observations for each group.

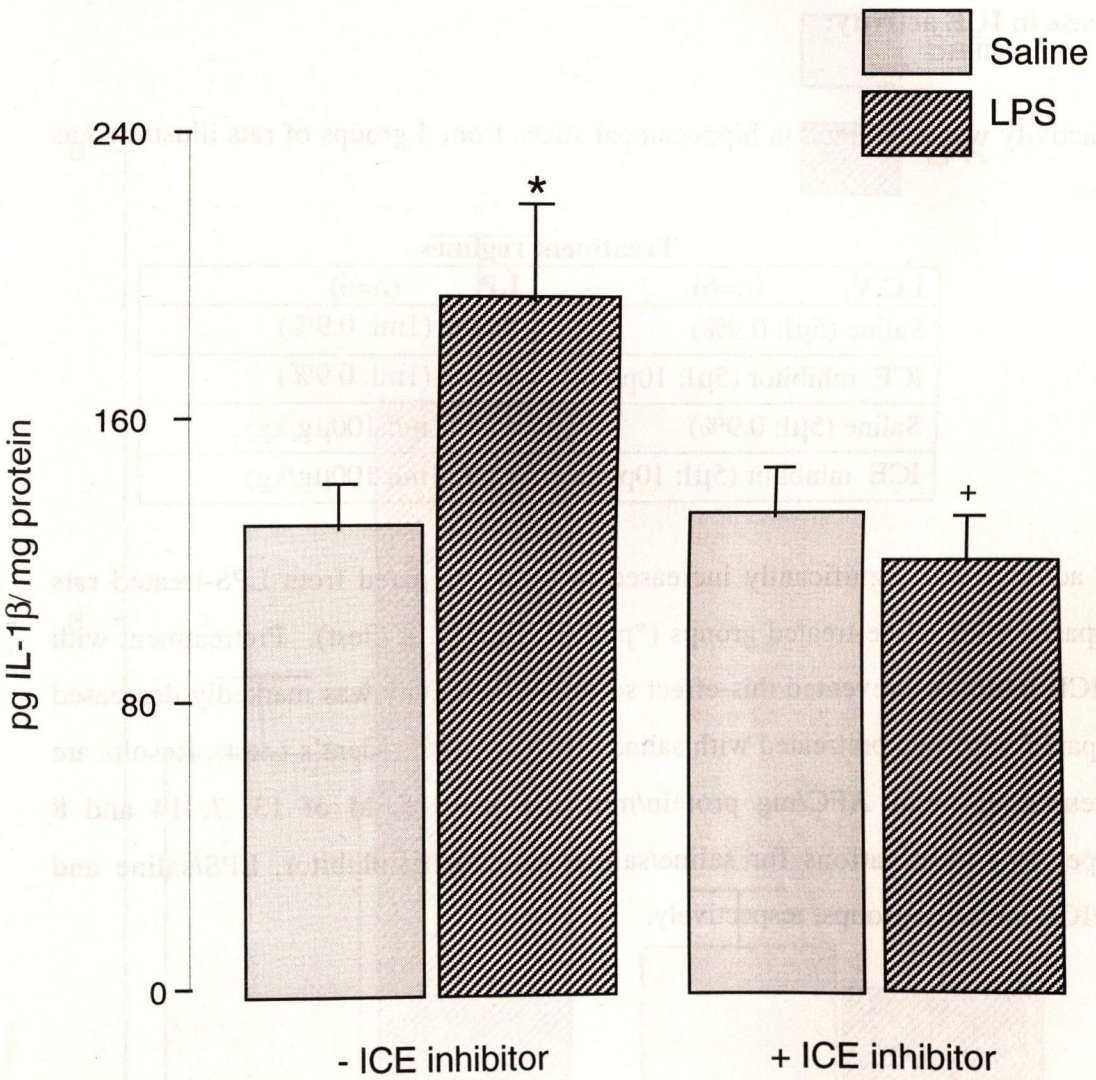

Saline
LPS
240
160
80
0
pg IL-1β/ mg protein
*
+
- ICE inhibitor
+ ICE inhibitor

Fig 6.10 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in ICE activity:

ICE activity was examined in hippocampal slices from 4 groups of rats illustrated as follows:

Treatment regimes

I.C.V. (n=6)	**I.P.** (n=6)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

ICE activity was significantly increased in tissue prepared from LPS-treated rats compared with saline-treated groups (*$p<0.05$; Student's t-test). Pretreatment with the ICE inhibitor prevented this effect so that ICE activity was markedly decreased compared with rats pretreated with saline (***$p<0.001$; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are ± SEM of 13, 7, 14 and 8 independent observations for saline/saline, saline/ICE inhibitor, LPS/saline and LPS/ICE inhibitor groups, respectively.

Fig 6.10 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in ICE activity:

ICE activity was examined in hippocampal slices from 4 groups of rats illustrated as follows:

Treatment regimes

I.C.V. (n=6)	**I.P.** (n=6)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

ICE activity was significantly increased in tissue prepared from LPS-treated rats compared with saline-treated groups (*$p<0.05$; Student's t-test). Pretreatment with the ICE inhibitor prevented this effect so that ICE activity was markedly decreased compared with rats pretreated with saline (***$p<0.001$; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are ± SEM of 13, 7, 14 and 8 independent observations for saline/saline, saline/ICE inhibitor, LPS/saline and LPS/ICE inhibitor groups, respectively.

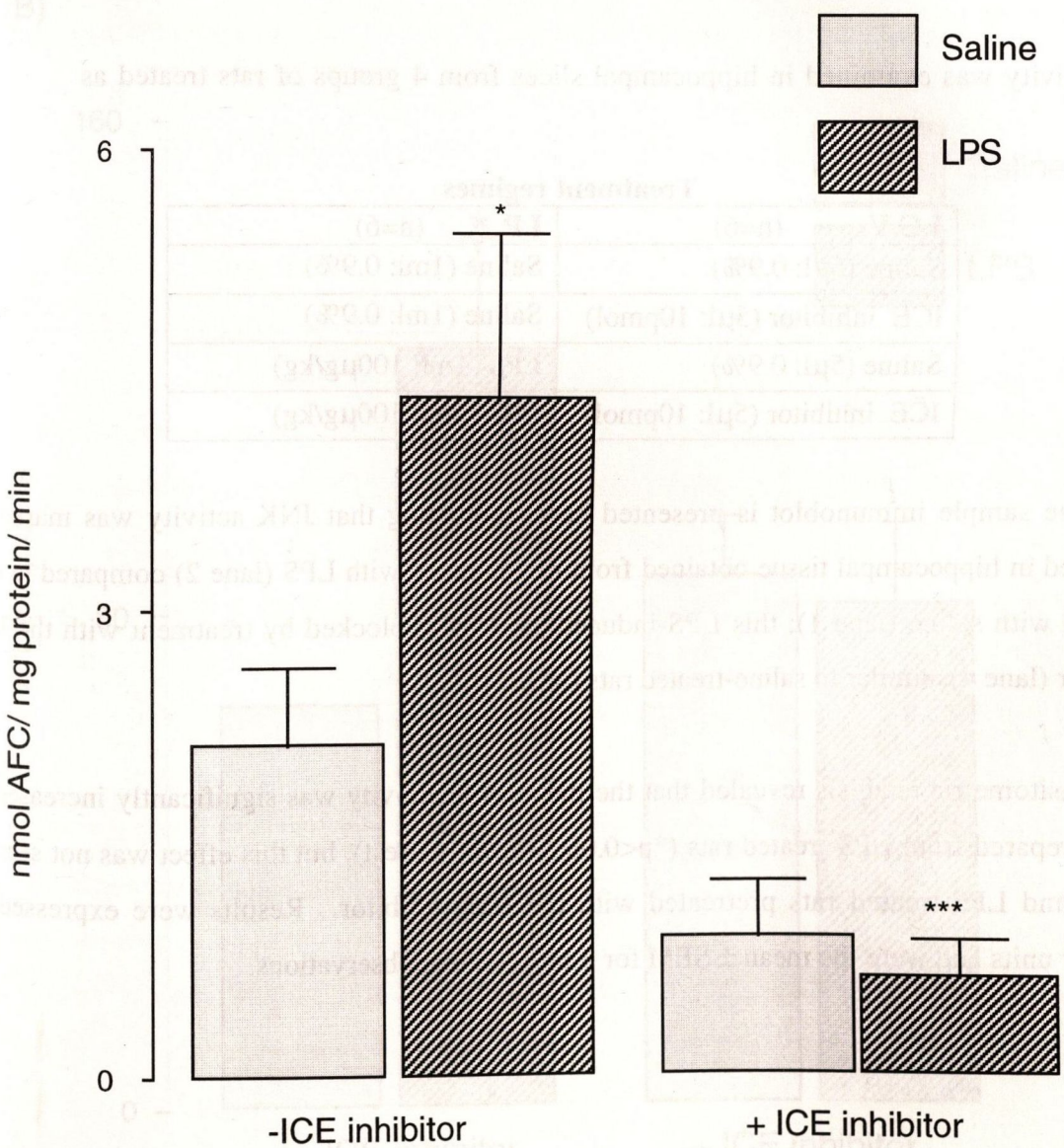

Saline
LPS
6
3
0
nmol AFC/ mg protein/ min
*
***
-ICE inhibitor
+ ICE inhibitor

Fig 6.11 Effect of the ICE inhibitor, Ac-YVAD-CMK, on the LPS-induced increase in JNK activity:

JNK activity was examined in hippocampal slices from 4 groups of rats treated as follows:

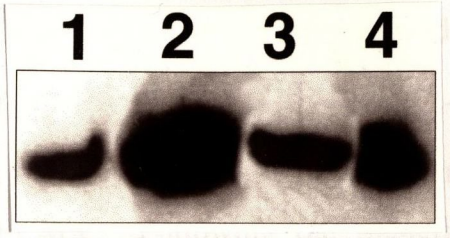
Treatment regimes

I.C.V. (n=6)	**I.P.** (n=6)
Saline (5µl: 0.9%)	Saline (1ml: 0.9%)
ICE inhibitor (5µl: 10pmol)	Saline (1ml: 0.9%)
Saline (5µl: 0.9%)	LPS (1ml: 100µg/kg)
ICE inhibitor (5µl: 10pmol)	LPS (1ml: 100µg/kg)

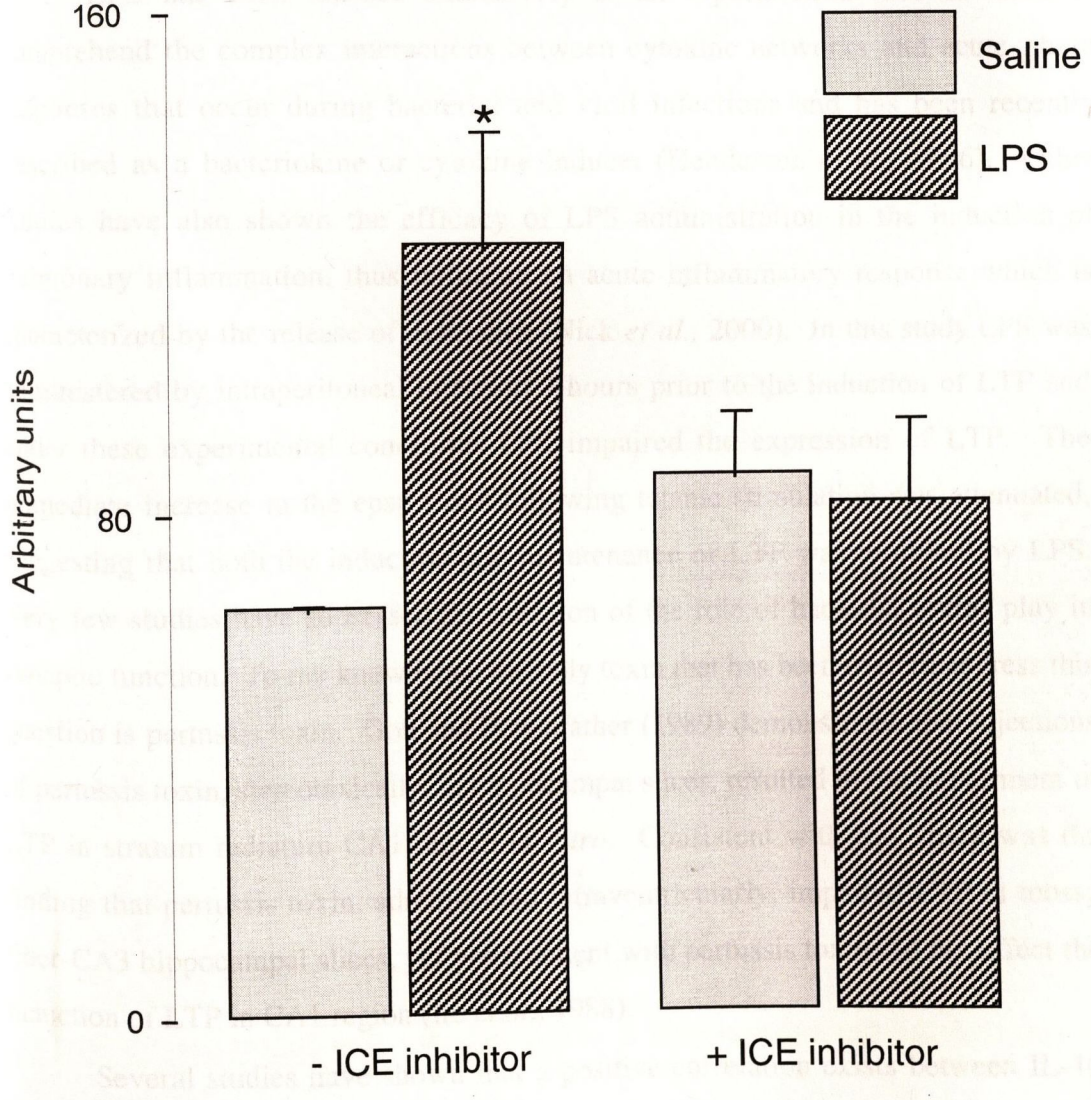
(A) One sample immunoblot is presented here, indicating that JNK activity was markedly increased in hippocampal tissue obtained from rats injected with LPS (lane 2) compared to rats injected with saline (lane 1); this LPS-induced effect was blocked by treatment with the ICE inhibitor (lane 4), similar to saline-treated rats (lane 3).

(B) Densitometric analysis revealed that the mean JNK activity was significantly increased in tissue prepared from LPS-treated rats (*$p<0.05$; student's t-test), but this effect was not seen in saline- and LPS-treated rats pretreated with the ICE inhibitor. Results were expressed as arbitrary units and were the mean ± SEM for 3 independent observations.

(A)


1
2
3
4

(B)


160
80
0
Arbitrary units
*
Saline
LPS
- ICE inhibitor
+ ICE inhibitor

6.4 Discussion:

The objective of this study was to investigate the effect of intraperitoneal injection of lipopolysaccharide (LPS) on LTP in the rat dentate gyrus *in vivo* and assess biochemical changes that underlie functional changes. Thus, glutamate release was assessed in parallel with IL-1β concentration, ICE, JNK and p38 activity and reactive oxygen species (ROS) formation. The possibility that IL-1β was primarily responsible for the LPS-induced changes was assessed by investigating the effect of the ICE inhibitor, Ac-YVAD-CMK. Following the induction of LTP, the concentration of IL-1β was assessed in parallel with the activity of ICE and JNK.

LPS has been utilized extensively as an experimental tool in order to comprehend the complex interactions between cytokine networks and acute phase responses that occur during bacterial and viral infections and has been recently described as a bacteriokine or cytokine inducer (Henderson *et al.,* 1996). Other studies have also shown the efficacy of LPS administration in the induction of pulmonary inflammation, thus initiating an acute inflammatory response which is characterized by the release of cytokines (Nick *et al.,* 2000). In this study LPS was administered by intraperitoneal injection 3 hours prior to the induction of LTP and under these experimental conditions LPS impaired the expression of LTP. The immediate increase in the epsp slope following tetanic stimulation was attenuated, suggesting that both the induction and maintenance of LTP was inhibited by LPS. Very few studies have addressed the question of the role of bacterial toxins play in synaptic function. To my knowledge, the only toxin that has been used to address this question is pertussis toxin. Goh and Pennefather (1989) demonstrated that injections of pertussis toxin, stereotaxically, in hippocampal slices, resulted in the impairment of LTP in stratum radiatum-CA1 region *in vitro*. Consistent with this study was the finding that pertussis toxin, administered intraventricularly, impaired LTP in mossy fiber-CA3 hippocampal slices, yet pretreatment with pertussis toxin did not affect the induction of LTP in CA1 region (Ito *et al.*, 1988).

Several studies have shown that a positive correlation exists between IL-1β and LPS, and a negative correlation exists between IL-1β and LTP. Thus LPS induces an increase in IL-1β concentration, for example, in macrophages and glia

(Loscher, *pers. comm*; Kong *et al.*, 1997). Moreover, peripheral administration of LPS induces a time- and dose-dependent increase in IL-1β immunoreactivity of IL-1β in rat meningeal and choroid plexus macrophages and microglial cells in the hypothalamus and cerebral cortex (Van Dam *et al.*, 1998). Consistent with this report, is the finding that subseptic and subfebrile doses of LPS, administered intravenously, activates the induction of IL-1β mRNA in rat circumventricular organs (CVOs) and in meningeal cells (Quan *et al.*, 1999). It has been widely reported that IL-1β impairs LTP *in vivo* and *in vitro,* suggesting a role for IL-1β in synaptic function. For example, IL-1β has been shown to inhibit LTP *in vitro* in CA1 (Bellinger *et al.*, 1993), CA3 (Katsuki *et al.*, 1990) and dentate gyrus (Cunningham *et al.*, 1996) while it also inhibited LTP in the dentate gyrus *in vivo* (Murray & Lynch, 1998a,b). From these reports it might be proposed that an LPS-induced increase in IL-1β in the hippocampus might lead to an inhibition of LTP.

LTP is associated with a concomitant increase in glutamate release from perforant-path granule cell synapses (Bliss & Collingridge, 1993; McGahon & Lynch, 1996, McGahon *et al.*, 1997) and therefore endogenous glutamate release was assessed in this study. It was found that KCl-induced depolarization caused a significant increase in glutamate release in synaptosomes prepared from tetanized dentate gyrus compared with untetanized dentate gyrus and these results confirm earlier reports which couple LTP with an increase in glutamate release (Reichter-Levin *et al.*, 1995; McGahon & Lynch, 1996). However, LPS treatment permitted the effect of KCl on glutamate release in synaptosomes prepared from both tetanized and untetanized dentate gyrus. The data show that LPS impacts negatively on synaptic function, since it attenuates both LTP and endogenous glutamate release. It is possible that an LPS-induced increase in IL-1β may lead to an inhibition of glutamate release. This hypothesis is consistent with previous evidence, indicating that IL-1β inhibits endogenous glutamate release in the rat dentate gyrus and hippocampus *in vitro* (Murray *et al.*, 1997).

In order to test the hypothesis that LPS increased IL-1β concentration in the hippocampus, as shown in other tissues (Loscher, personal communication; Iiyin *et al.*,

1998), IL-1β expression was assessed in dentate gyrus synaptosomes. The data indicated that IL-1β concentration was increased in both untetanized and tetanized dentate gyrus prepared from LPS-treated rats compared with saline-treated rats and there was no significant difference between IL-1β concentrations in untetanized and tetanized tissue in both cases. Since IL-1β has been shown to decrease glutamate release *in vitro* (Murray *et al.,* 1997), the present data are consistent with the hypothesis that LPS might compromize synaptic plasticity in rat dentate gyrus by inducing an increase in IL-1β concentration.

IL-1β must be cleaved to be activated, and this event is achieved by IL-1β converting enzyme (ICE), a cysteine protease which cleaves the 30 kD pro-IL-1β to its mature 17kDa form, allowing release of active IL-1β during tissue insult and injury (Thornberry *et al.,* 1992). Since ICE is necessary for the activation of IL-1β, the effect of LPS administration on ICE activity was assessed in rat hippocampal tissue. The results revealed an LPS-induced increase in ICE activity, which correlates with the LPS-induced increase in IL-1β concentration. This finding is consistent with other studies where ICE activity was increased upon bacterial infection. This is exemplified in studies undertaken by Schumann and colleagues (1998), who demonstrated that ICE activity was increased in cultured human monocytic and human umbilical cord endothelial cells upon treatment with LPS. It was also determined from these studies that the LPS-induced ICE activity was concentration- and time-dependent and was associated with an increase in IL-1β concentration (Schumann *et al.,* 1998). A similar finding has also been documented in human monocyte-derived macrophages infected with *Shigella flexneri*, a causative agent of bacillary dysentary (Hilbi *et al.,* 1997). An LPS-induced increase in ICE mRNA has been shown in NI3 and primary microglial cells, but not in microglia obtained from endotoxin resistant C3H/HeOuJ mice (Yoa *et al.,* 1997). However, the LPS-induced increase in ICE activity presented here and previously documented, is in contrast to a previous finding in which ICE activity was triggered in pituitary gland, but not in the hippocampus or hypothalamus, although mRNA for ICE was increased in all regions (Tingsborg *et al.,* 1996), suggesting the ICE activity is specific for specific regions of the brain.

This study assessed the effect of LPS treatment on JNK and p38 activity in hippocampal tissue, and the results revealed an LPS-induced increase in the activity of these kinases. Similar effects have been reported in other cell types, such as neutrophils exposed to LPS (Nick *et al.,* 1996), whereby activation of p38 occurred in a dose-dependent manner. The same group reported that p38 activity was increased in LPS-treated human and murine macropahges, and this effect was inhibited by a specific inhibitor of p38, M39 (Nick *et al.,* 2000). Consistent with these reports and the data presented here, is the finding that both p38 and JNK activity were enhanced 5- to 10-fold in LPS-treated murine macrophages *in vitro* (Sanghera *et al.,* 1996). It could be speculated that the LPS-induced increase in JNK and p38 activity described here maybe mediated by IL-1β, since previous *in vitro* studies indicate that IL-1β stimulated activation of p38 and JNK activity.

Previous findings have shown that IL-1β can induce reactive oxygen species (ROS) formation in the rat hippocampus (O'Donnell et al., 2000) and cortex (O'Donnell & Lynch, 1998). On the basis of these findings the effect of LPS on ROS was assessed in hippocampal synaptosomes. The data reveal that ROS formation was increased in hippocampal synaptosomes prepared from LPS-treated rats compared with hippocampal synaptosomes prepared from saline-treated rats. Since LPS induced an increase in IL-1β expression in the hippocampus, ROS formation may be secondary to this effect, as proposed by O'Donnell *et al* (2000). As previously suggested, the LPS-induced increase in ICE activity may play an important role in inhibiting synaptic function. The finding that LPS also increase the formation of ROS, may suggest a relationship between ROS formation and ICE activity. Caspases, such as ICE, are cysteine-dependent enzymes and appear to be redox sensitive and, depending on the efficacy of redox activity, either induction or inhibition of caspase activity may transpire (see Hampton *et al.,* 1998).

If the LPS-induced increase in IL-1β concentration and/or ICE and JNK activity are primarily responsible for the inhibition of LTP and glutamate release, then it follows that LTP would not be affected if these changes were inhibited. Similarly, if IL-1β mediates the LPS-induced effects, through the activity of ICE, it might be predicted that pre-treatment with a peptide ICE inhibitor, Ac-YVAD-CMK, might

impede the LPS-induced inhibitory effects. The present findings show that pre-treatment with the peptide ICE inhibitor blocked the inhibitory effect of LPS on the expression of LTP. In parallel with this finding, it was established that the LPS-induced increase in the concentration of IL-1β, and the activities of ICE and JNK were blocked. This result suggests that a causal link exists between ICE activity and LPS, supporting the proposal that increased IL-1β concentration, coupled with the consequent increase in JNK activity, maybe responsible for the impairment in LTP (as discussed in previous chapters). In the context of these findings, it is significant that the employment of a suitable ICE inhibitor is being considered as an appropriate intervention in the inhibition of neurotoxicity (Hilbi *et al.*, 1997).

The induction of ICE activity has been associated with apoptosis or programmed cell death when murine Hepa 1c1c7 hepatoma cells are infected with the apoptotsis-inducing agent, Benzo(a)pyrene (Lei *et al.*, 1998). Similarly, the activation of ICE has been considered a contributory factor in the induction of the cell damage associated with ischemia in mouse brain (Frielander *et al.*, 1997). In this study, a mutant ICE gene was designed to act as a dominant negative ICE inhibitor, and dorsal root ganglial neurons obtained from these transgenic mice were unable to resist trophic factor withdrawal-induced apoptosis. These findings suggest a role for ICE in apoptosis, and a role for ICE inhibitors in modulating ICE-associated changes in tissue insult (Frielander *et al.*, 1998). The results obtained from this study are largely consistent with these findings, in that inhibition of ICE activity reversed LPS-induced deterioration of synaptic function.

The data presented here demonstrate that when LPS is administered intraperitoneally, LTP and the concomitant increase in glutamate release are impaired. The evidence suggests that these changes are associated with concomitant increase in the expression of IL-1β, ICE activity and activity of the stress activated protein kinases, JNK and p38. It appears that an increase in IL-1β concentration maybe the primary cause of these LPS-induced changes, as the peptide ICE inhibitor, Ac-YVAD-CMK, reversed these effects.

Chapter 7

The effect of lipopolysaccharide on synaptic function and apoptotic cell death: A role for p38 kinase?

7.1 Introduction:

Gram-negative bacteria are associated with multiple pathophysiological changes and it has been generally accepted that lipopolysaccharide (LPS), a component of the outer membrane of most gram-negative bacteria, is responsible for these changes (Marsh *et al.,* 1996). It is thought that these changes are mediated by interleukin-1β (IL-1β), as LPS increases IL-1β concentrations (Ilyin *et al.,* 1998) and IL-1β mRNA expression (Quan *et al.,* 1999). This sequence of events is tightly regulated because over-expression of immune mediators can result in tissue injury and neuronal deficits (Marsh *et al.,* 1996). A prime example of one neuronal deficit due to IL-1β is inhibition of long-term potentiation (LTP) *in vitro* (Katsuki *et al.,* 1990; Bellinger *et al.,* 1993; Cunningham *et al.,* 1996) and *in vivo* (Murray & Lynch, 1998a; 1998b; O'Donnell *et al.,* 2000). It could be predicted that a potent stimulus such as LPS mediates its effects through increased IL-1β production.

Interleukin-1 converting enzyme (ICE) or caspase-1, functions primarily by cleaving the inactive precursor, pro-IL-1β, to the mature and active form, IL-1β (Thornberry *et al.,* 1992). LPS activates ICE in a variety of cell types, such as cultured monocytes and endothelial cells (Schumann *et al.,* 1998) and human monocyte-derived macrophages (Hilbi *et al.,* 1997). Further confirmation of LPS-induced ICE activity has stemmed from studies on transgenic mice which express a dominant negative mutant of ICE (Frielander *et al.,* 1997) and in studies utilizing the ICE-like protease inhibitors, z-VAD.FMK (Hara *et al.*, 1997). In both studies, data indicate that the inhibition of ICE reduces ischemic and excitotoxic neuronal damage. It might therefore be suggested that ICE activation plays a role in LPS-induced impairments.

Apoptotic cell death is a fundamental and natural biological process that aids maintenance of cell homeostasis, and the apoptotic cell death pathway is essentially controlled by cysteine proteases, known as caspases (Nicholson & Thornberry, 1997). In addition to ICE, another and more prominent marker of cell death is caspase-3/CPP32, a key executioner of apoptosis which is widely distributed in lymphocytic-derived cells (Nicholson *et al.,* 1995), cervical ganglion neurons (McCarthy *et al.,* 1997) and rat hepatocytes (Hamada *et al.,* 1999).

Recently, the possibility of a relationship between apoptosis, caspases and stress-activated protein kinases has been suggested. In neuronally differentiated

PC12 cells, NGF withdrawal induces apoptosis, and this is accompanied by increased activity of JNK and p38 (Xia *et al.*, 1995). However, various studies have addressed the possibility that the effects of p38 and apoptosis may occur in a concomitant manner. p38 has recently been implicated in mediating apoptosis in several cell types in various species, such as in rat retinal ganglion cells (Kikuchi *et al.*, 2000) and rat cerebellar granule cells (Kawasaki *et al.*, 1997). One of the downstream effects of p38 activity is the activation of transcription factors. It has been recently shown that p38 is necessary for the activation of nuclear factor kappa-B (NF-κB) transcription factor (Carter *et al.*, 1999), which has been implicated in synaptic plasticity (Meberg *et al.*, 1996), glial and neuronal cell function (Heyen *et al.*, 2000) and apoptosis (Lang *et al.*, 2000).

The primary aim of this study was to further elucidate the effect of LPS on synaptic function by specifically investigating neuronal viability and to determine whether or not increased p38 activity plays a role in LPS-induced changes. The effect of LPS on p38 activity was assessed, and in parallel, the effect of SB 203580 on LPS- and IL-1β-induced inhibition of LTP and glutamate release was analysed. The activity of ICE and concentration of IL-1β was investigated to determine if they played a role in the inhibitory effects of LPS. In addition, to determine the extent of LPS-induced damage, markers of apoptosis, such as caspase-3 activity and morphological changes were analysed.

7.2 Methods

7.2.1 Treatment regimes

Five groups of Wistar rats were anaesthetized intraperitoneally (i.p.) with urethane (1.5g/kg). The head was positioned in a head holder within a sterotaxic frame. Two of the groups were injected i.p. with either saline (1ml) or LPS (1ml: 500μg/kg) 3 h prior to the induction of LTP. A third group was injected intracerebroventricularly (i.c.v.) with IL-1β (3.5ng/ml) prior to the induction of LTP. The last two groups were injected i.c.v. (section 2.5.4) with SB 203580 (50μM) and then injected with either LPS (i.p.) or IL-1β (3.5ng/ml: i.c.v.) 3 h prior to the induction of LTP. The injection protocol is illustrated in table 1.

Table 1.

	I.C.V. (n=6)	**I.P. (n=6)**
1.		Saline (1ml: 0.9%)
2.		LPS (1ml: 500μg/kg)
3.	IL-1β (10μl: 3.5ng/ml)	
4.	SB 203580 (10μl: 50μM)	LPS (1ml: 500μg/kg)
5.	SB 203580 (10μl: 50μM) IL-1β (10μl: 3.5ng/ml)	

7.2.2 Induction of LTP in vivo

Following i.p. and i.c.v. injections, rats were positioned in a sterotaxic frame and a window of skull was removed to allow the insertion of the stimulating and recording electrodes in the perforant path and granule layer of the dentate gyrus (see section 2.5.2 and 2.5.3). The depths of the electrodes were adjusted to obtain maximal responses. Electrophysiological recording commenced 30 min after the i.c.v and i.p. injections. Test shocks, at a rate of 1/30 sec, were delivered for 10 min prior to, and 40 min after, tetanic stimulation (3 trains of stimuli; 250 Hz for 200 msec; 30 sec intertrain interval).

7.2.3 Tissue storage

At the end of the electrophysiological recording period, rats were killed by cervical dislocation (section 2.3). The hippocampus was removed and the untetanized

and tetanized dentate gyri, as well as hippocampus proper, were dissected on ice and prepared for storage as described (see section 2.3.1).

7.2.4 Analysis of p38 activity

The activity of p38 was assessed only in the saline- and LPS-treated groups in P_2 preparations from hippocampal slices obtained from both saline- and LPS-treated groups. The analysis of p38 was assessed as described in section 2.8.1 and 2.8.2. Briefly, proteins were separated by gel electrophoresis, transferred onto nitrocellulose paper and immunoblotted with an anti-phospho p38 antibody (section 2.8.3). The antibody complex was visualized using ECL detection and protein bands were quantified by densitometric analysis and results were expressed as arbitrary units.

7.2.5. Analysis of glutamate release *in vivo*

The impure synaptosomal preparation, P_2, was prepared from untetanized and tetanized dentate gyrus as described (see section 2.3.2). The synaptosomal pellet was resuspended in Krebs solution containing $CaCl_2$ (final concentration: 2mM). For *in vitro* analysis, synaptosomes were preincubated for 20 min in oxygenated KrebsCa containing either IL-1β (final concentration: 10pg/ml) or SB 203580 (final concentration: 50μM). Briefly, synaptosomes were aliquotted onto a Millipore filtration manifold, washed under vacuum and release was assessed in the absence or presence of 50mM KCl. Filtrates were collected and analysed by immunoassay (section 2.7). Results were expressed as μmol glutamate/mg protein.

7.2.6 Analysis of Interleukin-1β concentration

The concentration of IL-1β was assessed in hippocampal homogenate prepared from saline- and LPS-treated rats (section 2.6.1). Slices were homogenized and protein concentrations was equalized (section 2.4) and aliquots were added to a 96-well microtiter plate coated with capture antibody and incubated for 1 h prior to the addition of the secondary antibody. The detection antibody reagent and the working substrate, TMB were added and the samples were read at 450nm (section 2.9). IL-1β concentration was expressed as pg IL-1β/mg protein.

7.2.7 Analysis of Interleukin1β Converting Enzyme (ICE) activity

Slices of hippocampus obtained from saline- and LPS-treated rats were homogenised in lysis buffer and put through 4 freeze-thaw cycles and centrifuged to provide supernatant. Aliquots were added to ICE substrate (10µl: YAVD peptide) and incubation continued for 1h at 37°C (see section 2.10) and the samples read at 505nm (emission) upon excitation at 400nm. Results were expressed as nmol AFC/mg protein/min.

7.2.8 Analysis of caspase-3 activity

Slices of hippocampus obtained from saline- and LPS-treated rats were homogenised in lysis buffer and put through 4 freeze thaw cycles and centrifuged to provide supernatant. Aliquots were added to caspase-3 substrate (10µl: DEVD peptide) and incubation continued for 1h at 37°C (see section 2.10) and the samples read at 505nm (emission) upon excitation at 400nm. Results were expressed as nmol AFC/mg protein/min.

7.2.9 Analysis of NF-κB transcriptional activity

The transcriptional activity of NF-κB was assessed in nuclear extracts prepared from dissociated entorhinal cortical cells harvested from saline- and LPS-treated rats. Suspensions of dissociated cells were prepared as described (section 2.13.1), and nuclear extracts were obtained (section 2.14.1) and equalized for protein (section 2.4). A [^{32}P] labelled DNA fragment containing the NF-κB motif was incorporated into the samples by incubation and samples were loaded onto 4% acrylamide gels (section 2.14.2). Gels were dried onto filter paper, exposed to film for one week and processed using a Fuji X-ray developer.

7.10 Analysis of degenerating or apoptotic cells: TUNEL assay

Dissociated cells were prepared from hippocampal and entorhinal cortical cells as described previously (section 2.13.1) and cytospun onto glass microscope slides and fixed in methanol and paraformaldehyde. The cells were permeabilized (section 2.13.2) and diaminobenzidine (DAB) was applied to the cells to stain apoptotic nuclei brown. Cells were washed and mounted with glycerine jelly. The number of cells stained from DAB was estimated as a % of the total.

7.11 Immunocytochemical analysis of p38 staining

Slices of entorhinal cortex were obtained from saline- and LPS–treated rats. Dissociated cells were prepared (section 2.13.1). Suspensions of dissociated cells were cytospun fixed in methanol and paraformaldehyde. Cells were incubated with a phosphospecific anti-p38 primary antibody and following incubation DAB was applied. Cells were counterstained in methylgreen, dehydrated in alcohol and mounted in DPX. The number of cells of positively stained cells were counted and expressed as a % of the total.

7.3 Results

7.3.1 Effect of LPS on p38 activity:

Activity of p38 was assessed from hippocampal tissue obtained from saline- and LPS-treated rats. The sample immunoblot illustrated in fig. 7.1 (A) demonstrates that p38 activity was increased in hippocampal tissue obtained from LPS-treated rats compared with saline-treated rats. The mean data obtained from densitometric analysis are shown in fig. 7.1 (B). The mean values, expressed in arbitrary units, show that p38 activity was significantly increased from 14.9 ± 1.3 (SEM; n=6) to 20.8 ± 2.4 in saline- and LPS-treated rats, respectively

7.3.2 Effect of LPS injection on IL-1β concentration

IL-1β concentration was assessed in hippocampal synaptosomes obtained from saline- and LPS-treated rats. The data illustrated in fig. 7.2 indicate that IL-1β concentration was significantly increased in hippocampal synaptosomes obtained from LPS-treated rats compared with saline-treated rats (*$p<0.05$; student's t-test). The mean values were 187.7 ± 34.0 pg/mg (SEM; n=6) and 261.3 ± 33.0 pg/mg, for saline and LPS-treated rats, respectively. .

7.3.3 Effect of LPS on ICE activity

The data indicate that ICE activity was significantly increased in hippocampal tissue prepared from LPS-treated rats compared with saline-treated rats (*$p<0.05$; student's t-test; fig. 7.3). The mean values were 0.42 ± 0.044 nmol/mg/min (SEM: n=7) and 0.677 ± 0.10 nmol/mg/min for saline- and LPS-treated rats, respectively.

7.3.4 Effect of SB203580 on IL-1β-induced inhibition of endogenous glutamate release

The effect of SB203580 (final concentration: 50μM) on the inhibitory effect of IL-1β (10pg/ml) on glutamate release in rat dentate gyrus was assessed. Fig. 7.4 indicates that KCl-stimulated release was significantly increased release in control synaptosomes (*$p<0.05$; student's t-test for paired values). Mean values were 0.65 ± 0.07 μmol/mg (SEM; n=13) and 0.92 ± 0.09 μmol/mg for unstimulated and KCl-stimulated release, respectively. Synaptosomes pretreated with IL-1β (10pg/ml) showed no increase in KCl-stimulated glutamate release compared to unstimulated release. The mean values were 0.87 ± 0.08 μmol/mg and 0.92 ± 0.13 μmol/mg for unstimulated and stimulated release, respectively. Synaptosomes preincubated in both SB203580 (50μM) and IL-1β (10pg/ml) showed a significant increase in KCl-stimulated glutamate release where the mean values were 0.82 ± 0.01 μmol/mg and 1.01 ± 0.10 μmol/mg for unstimulated and KCl-stimulated release respectively (+$p<0.05$; student's t-test).

7.3.5 Effect of SB 203580 on the IL-1β-induced impairment of LTP

Three pretreated groups of rats were analysed for the expression of LTP. Two of the groups were injected with either saline (1ml: 0.9%; i.p.) or with IL-1β (10μl; 3.5ng/ml: i.c.v) 3 h prior to the induction of LTP. The third group was injected i.c.v. with IL-1β (3.5ng/ml) and SB203580 (10μl; 50μM) prior to the induction of LTP. Fig 7.5 indicates that while IL-1β inhibited the expression of LTP, this effect was blocked by SB203580. The mean percentage change in epsp slope in the last 2 min immediately following tetanic stimulation was 160.5 ± 4 %(SEM: n=6) in the saline-treated compared with 120.3 ± 3.1 % in the group treated with IL-1β. In the last 5 min of the experiment the values were 145.7 ± 2.8 % and 106.2 ± 1.3 % respectively. The data indicates that IL-1β inhibited the expression of LTP in perforant-path granule cell synapses, however injection of SB203580 reversed the IL-1β-induced inhibition of LTP. The mean percentage changes were 175.6 ± 10.5 % and 164.97 ± 6.32 % in the first 2 min after tetanic stimulation and in the last 5 min of the experiment respectively.

7.3.6 Effect of SB203580 on the LPS-induced impairment of LTP

Three pretreated groups of rats were analysed for the expression of LTP. Two of the groups were injected i.p. with either saline (1ml: 0.9%) or LPS (1ml: 500μg/kg) 3 h prior to the induction of LTP. The third group was injected i.p. with LPS and i.c.v. with SB203580 (50μM) prior to the induction of LTP. Fig 7.6 indicates that while LPS inhibited the expression of LTP, this effect was blocked by SB203580. The mean percentage change in epsp slope in the last 2 min immediately following tetanic stimulation was 160.5 ± 4.1 % (SEM: n=6) in the saline-treated compared with 109.95 ± 4.3 % in the group treated with LPS. In the last 5 min of the experiment the values were 145.7 ± 2.8 % and 91.74 ± 1.65 % respectively. The data indicates that LPS inhibited the expression of LTP in perforant-path granule cell synapses, however injection of SB203580 reversed the LPS-induced inhibition of LTP. The mean percentage changes were 186.81 ± 12.05 % and 214.9 ± 15.3 % in the first 2 min after tetanic stimulation and in the last 5 min of the experiment respectively.

7.3.7 Effect of SB203580 on the IL-1β-induced inhibition of glutamate release following LTP

Glutamate release was assessed in synaptosomes prepared from tetanized and untetanized dentate gyrus obtained from rats injected intracerebroventricularly with SB203580 (50μM) and IL-1β (3.5ng/ml). Fig 7.7 indicates that the addition of 50mM KCl to synaptosomes prepared from untetanized dentate gyrus markedly increased glutamate release in the pretreated rats, but this result did not reach statistical significance. The mean values were 0.04 ± 0.012 μmol/mg (SEM; n=6) and 0.078 ± 0.012 μmol/mg, for unstimulated and KCl-stimulated glutamate release, respectively. The addition of 50mM KCl caused a further significant increase in glutamate release in synaptosomes prepared from tetanized dentate gyrus of the pretreated rats (**$p<0.01$; student's t-test). The mean values were 0.035 ± 0.0096 μmol/mg and 0.097 ± 0.012 μmol/mg, for unstimulated and KCl-stimulated glutamate release, respectively.

7.3.8 Effect of SB203580 on the LPS-induced inhibition of glutamate release following LTP

Glutamate release was assessed in synaptosomes prepared from tetanized and untetanized dentate gyrus obtained from rats injected intracerebroventricularly with

SB203580 (50μM) and LPS (500μg/kg). Fig 7.8 indicates that the addition of 50mM KCl to synaptosomes prepared from untetanized dentate gyrus markedly increased glutamate release in the pretreated rats, but this result was not statistically significant. The mean values were 0.046 ± 0.01 μmol/mg (SEM; n=6) and 0.066 ± 0.0212 μmol/mg, for unstimulated and KCl-stimulated glutamate release, respectively. The addition of 50mM KCl caused a further marked increase in glutamate release in synaptosomes prepared from tetanized dentate gyrus of the pretreated rats, but this result was not statistically significant. The mean values were 0.035 ± 0.011 μmol/mg and 0.107 ± 0.044 μmol/mg, for unstimulated and KCl-stimulated glutamate release, respectively.

7.3.9 Immunocytohistochemical analysis of p38 activity in entorhinal cortical cells:

The effect of saline and LPS treatment on p38 activity was assessed in entorhinal cortical cells by immunocytochemical analysis. Fig 7.9(A) illustrates that p38-positive cells are more abundant in entorhinal cortical cells obtained from LPS-treated (see picture A-II, indicated by black arrows) rats compared to saline-treated rats (see picture A-I). The number of p38-positive cells were counted, 100-200 cells per slide, and the mean data (fig. 7.9 (B)) revealed that the % p38-positive cells were significantly greater in entorhinal cortical cells obtained from LPS-treated rats than saline-treated rats (*$p<0.05$; student's t-test). The mean values were 6.97 ± 1.34 % and 50.40 ± 6.62 % (SEM; n=6) for saline- and LPS-treated rats, respectively.

7.3.10 Effect of LPS on the transcriptional activity of NF-κB:

Transcriptional activity of NF-κB was assessed in nuclear extracts prepared from dissociated cells obtained from saline- and LPS-treated rats. The sample immunoblot (fig. 7.10) illustrates that the activity of NF-κB was increased in nuclear extracts prepared from LPS-treated rats compared to nuclear extracts prepared from saline-treated rats. The sample immunoblot illustrates NF-κB activity in pairs (1-6), for saline- and LPS-treated rats, respectively, and the data indicate that an increase in NF-κB activity occurs in LPS-treated rats, in pair numbers 1, 2, 5 and 6.

7.3.11 Effect of LPS on caspase-3 activity:

The data indicate that caspase-3 activity was significantly increased in hippocampal tissue prepared from LPS-treated rats compared with saline-treated rats (**$p<0.01$; student's t-test; fig. 7.11). The mean values were 0.078 ± 0.023 nmol/mg/min (SEM: n=6) and 0.28 ± 0.05 nmol/mg/min for saline- and LPS-treated rats, respectively.

7.3.12 Effect of LPS on cell viability in hippocampal cells:

Apoptotic cell death was assessed in dissociated hippocampal cells prepared from saline- and LPS-treated rats by TUNEL assay. Fig. 7.12(A) illustrates that apoptotic cells prepared from LPS-treated rats (see picture A-I) were more abundant than cells prepared from saline-treated rats (see picture A-II). The number of degenerating cells were counted, 200-300 cells per slide, and the mean data (fig. 7.9 (B)) revealed that the % degenerating hippocampal cells was significantly greater in LPS-treated rats than in saline-treated rats (**$p<0.01$: student's t-test). The mean values were 27.0 ± 8.13 % (SEM: n=6) and 52.94 ± 13.91 % for saline- and LPS-treated rats, respectively.

7.3.13 Effect of LPS on cell viability in entorhinal cortical cells:

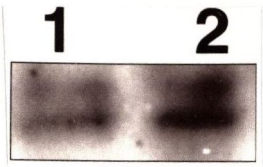
Apoptotic cell death was assessed in dissociated entorhinal cortical cells prepared from saline- and LPS-treated rats by TUNEL assay. Fig. 7.13(A) illustrates that apoptotic cells prepared from LPS-treated rats (see picture A-I) were more abundant than cells prepared from saline-treated rats (see picture A-II). The number of degenerating cells were counted, 200-300 cells per slide, and the mean data (fig. 7.13 (B)) revealed that the % degenerating entorhinal cortical cells were significantly greater in LPS-treated rats than in saline-treated rats (*$p<0.05$: student's t-test). The mean values were 24.4 ± 6.3 % (SEM: n=5) and 43.2 ± 2.9 % (SEM: n=6) for saline- and LPS-treated rats, respectively.

Fig. 7.1 Effect of LPS on p38 activity:

(A) One sample immunoblot is presented here, indicating that p38 activity was increased in hippocampal synaptosomes from LPS-treated (500μg/kg) rats (Lane 1) compared to that of saline-treated rats (Lane 2).

(B) Analysis of densitometric data indicates that the mean values for p38 phosphorylation was significantly greater in hippocampus from LPS-treated rats compared with saline-treated rats (*$p<0.05$; Student's t-test). Results are expressed in arbitrary units and are the means ± SEM of 6 independent observations.

(A)



(B)

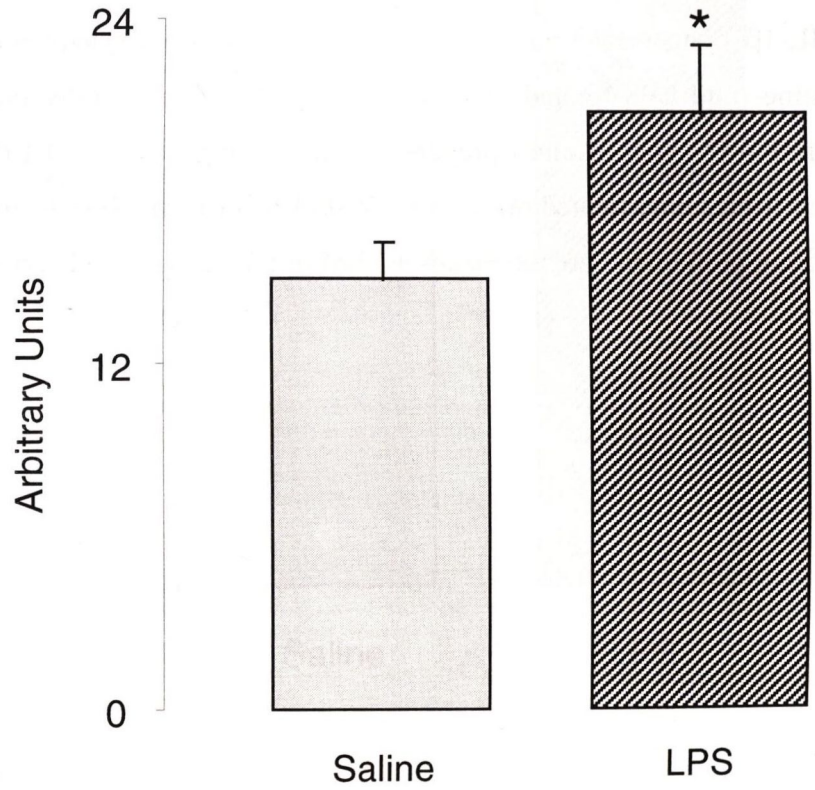


Fig.7.2 Effect of LPS injection on IL-1β concentration:

IL-1β concentration was analysed in hippocampal synaptosomes prepared from saline- and LPS-treated rats. LPS (500μg/kg) significantly increased IL-1β concentration in synaptosomes prepared from the hippocampus of LPS-treated rats compared from saline-treated rats (*$p<0.05$; student's t-test). Results are expressed as pg IL-1β/mg protein and are the means ± SEM of 6 independent observations.

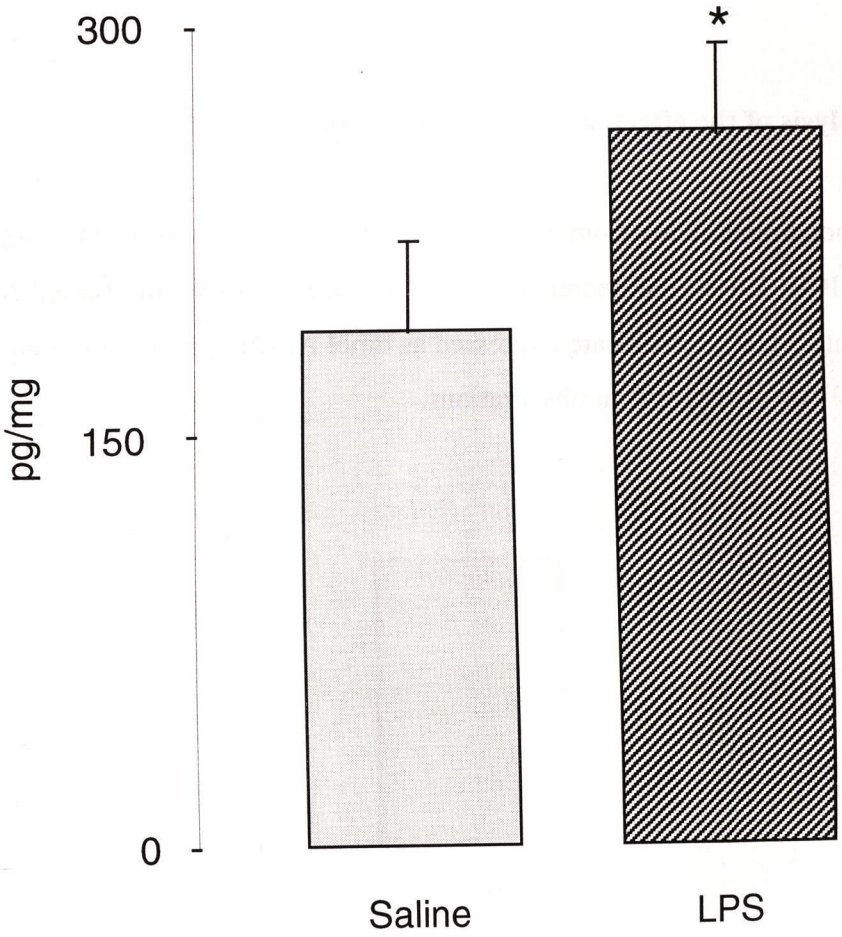

300
150
0
pg/mg
*
Saline
LPS

Fig 7.3 Analysis of the effect of LPS on ICE activity:

Hippocampal slices from LPS-treated (500μg/kg) rats showed a significant increase in ICE activity compared with that of slices from saline-treated rats (*p< 0.05; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are the means ± SEM of 7 independent observations.

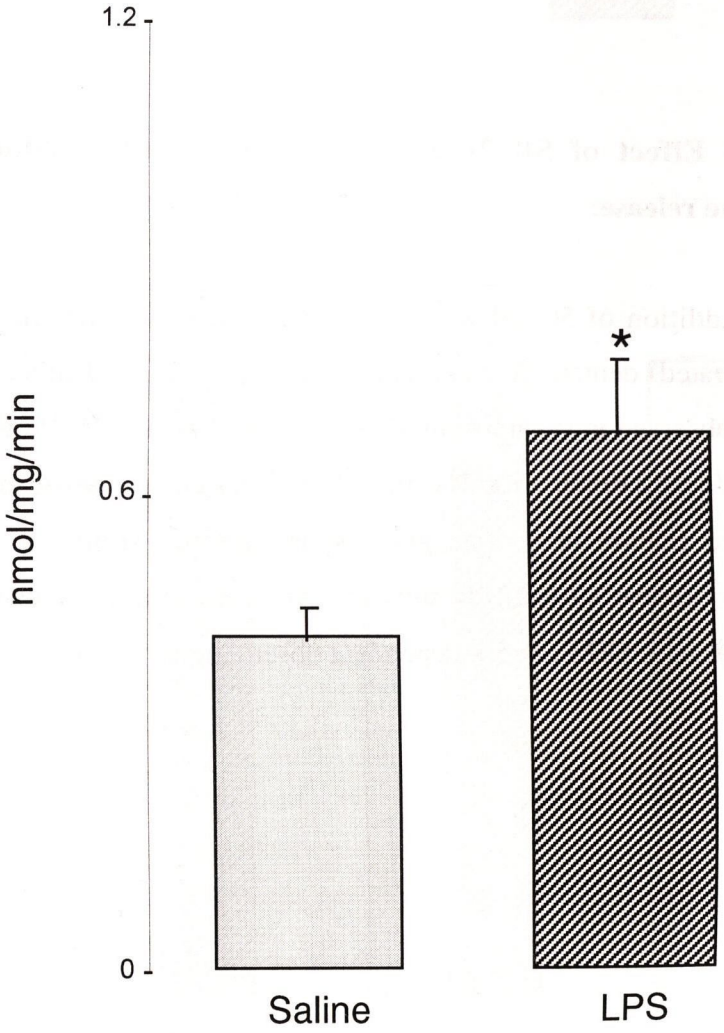
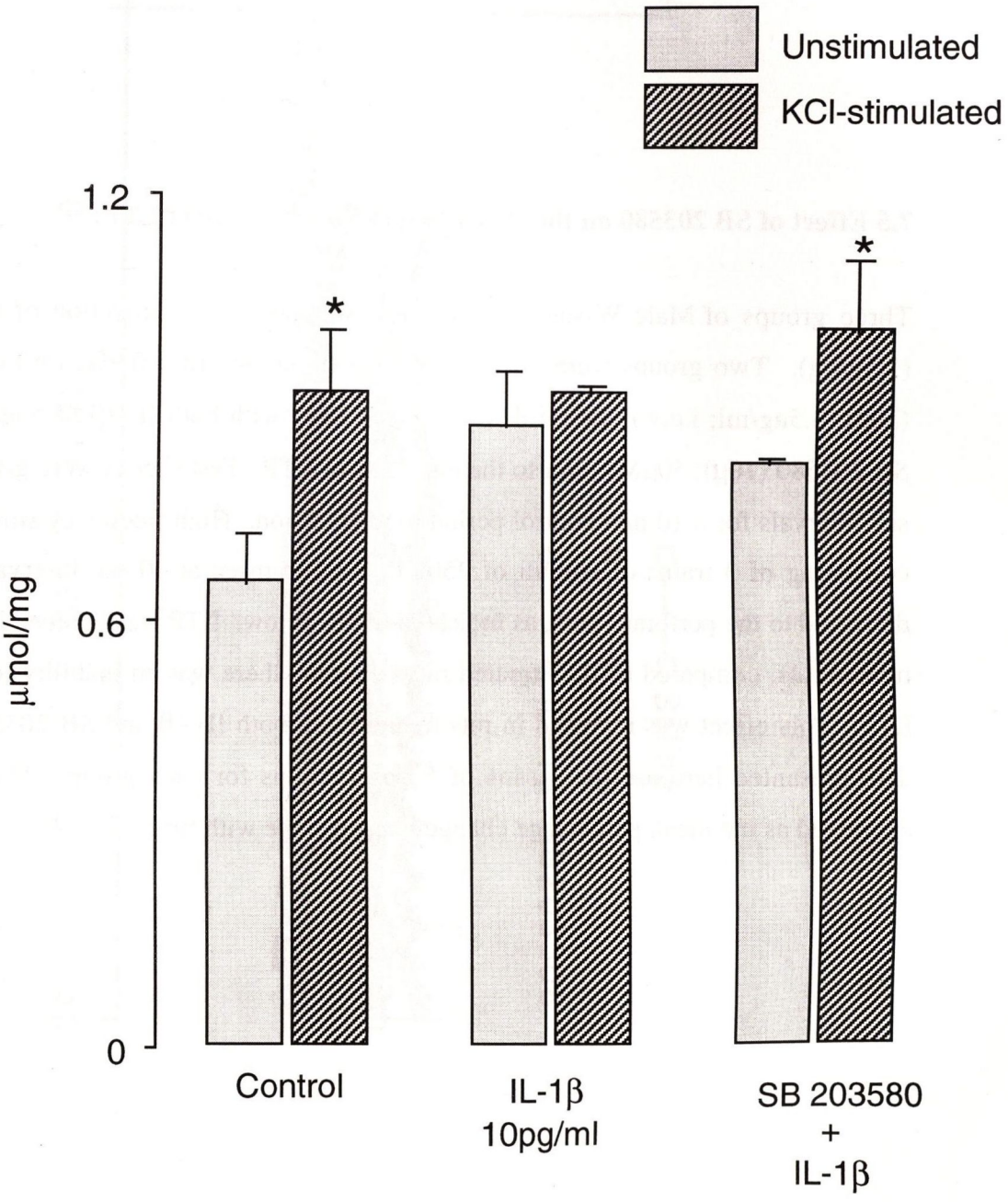

1.2
0.6
0
nmol/mg/min
*
Saline
LPS

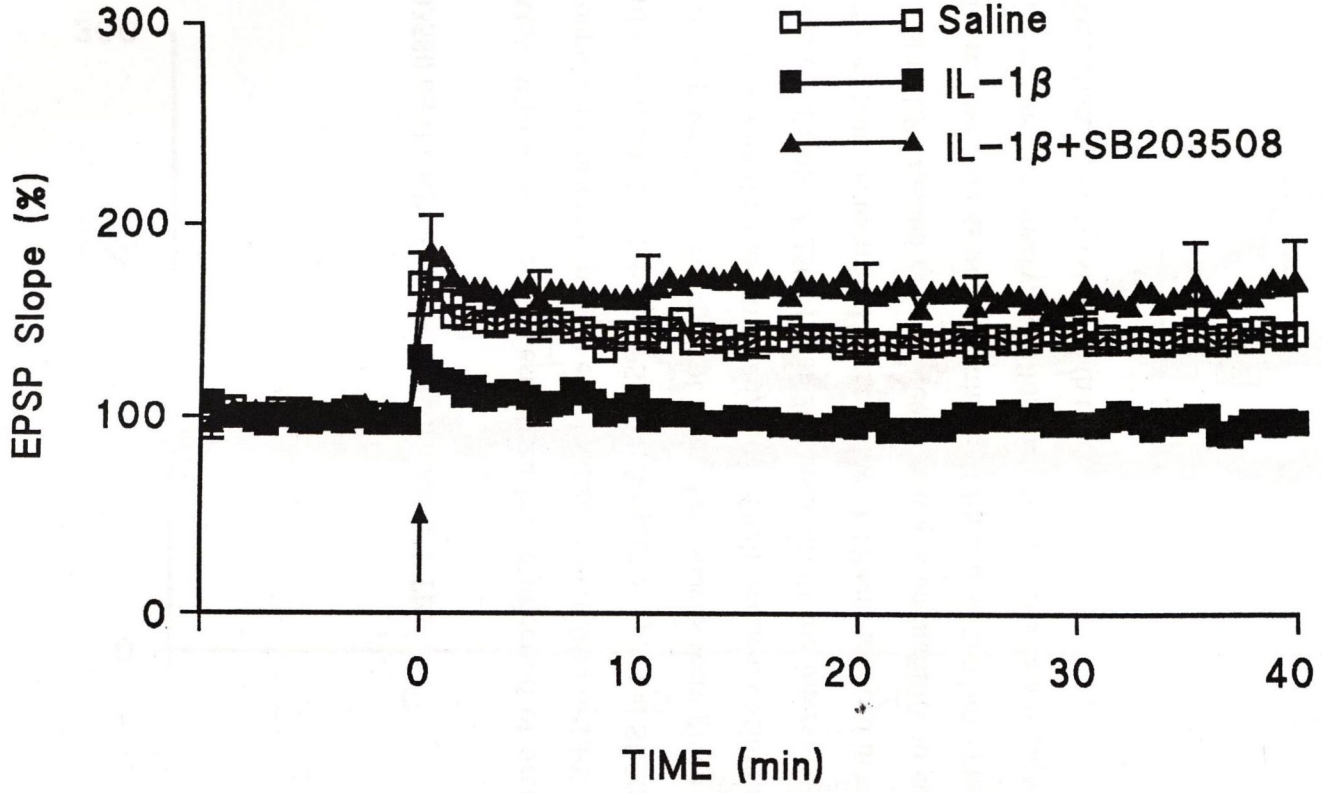
Fig. 7.4 Effect of SB 203580 on IL-1β-induced inhibition of endogenous glutamate release:

Addition of 50mM KCl significantly increased glutamate release in control (i.e. untreated) dentate gyrus synaptosomes (*$p<0.05$; student's t-test), but this effect was not observed in synaptosomes preincubated with IL-1β (10pg/ml). Preincubation with both SB230580 (50μM) and IL-1β caused a significant increase in KCl-stimulated release in dentate gyrus synaptosomes, similar to that of the control (*$p<0.05$; student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 13 independent observations.


Unstimulated
KCl-stimulated
1.2
0.6
0
µmol/mg
*
*
Control
IL-1β
10pg/ml
SB 203580
+
IL-1β

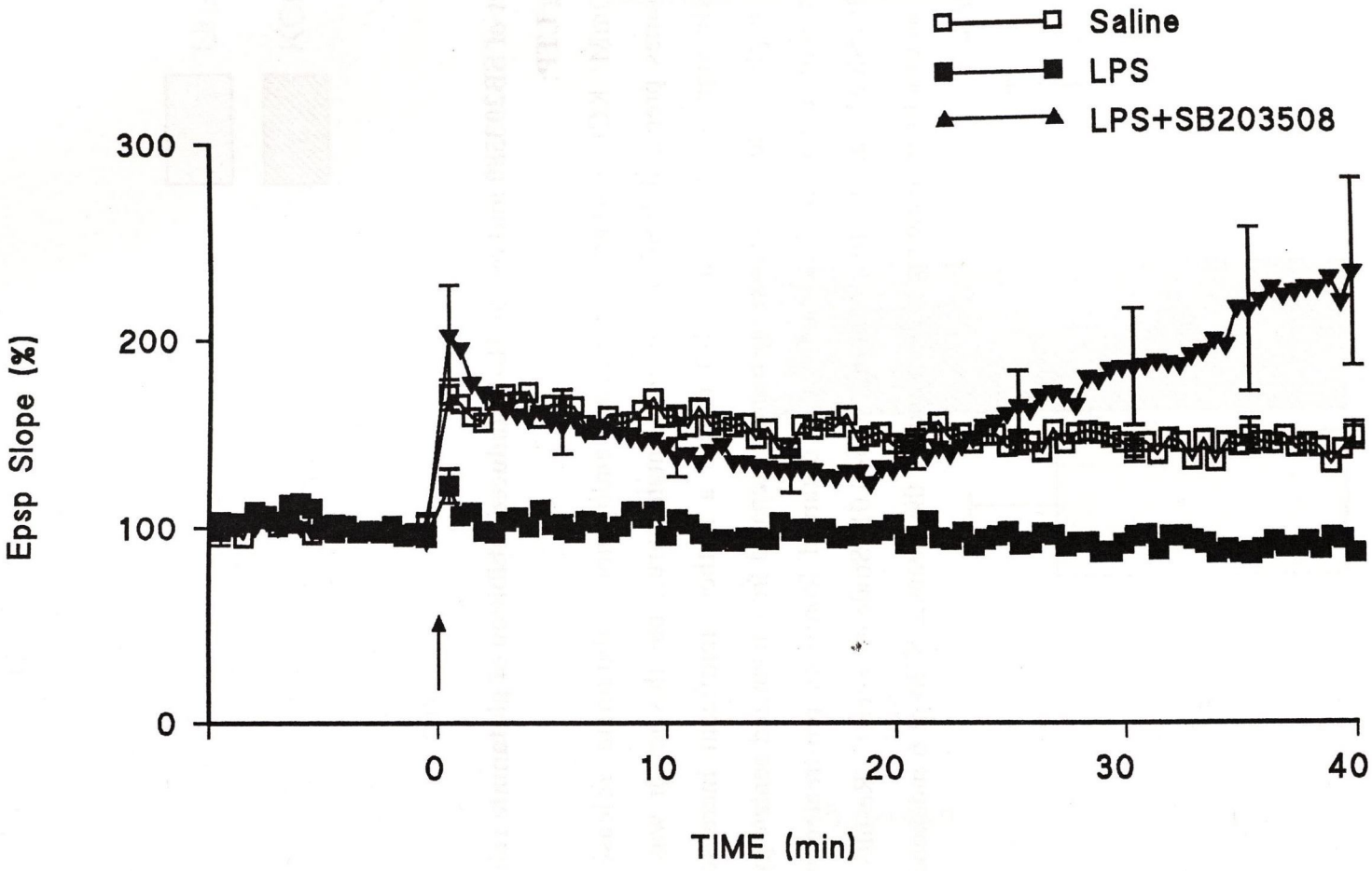
7.5 Effect of SB 203580 on the IL-1β-induced impairment of LTP:

Three groups of Male Wistar rats were anaesthetized by i.p injection of urethane (1.5g/kg). Two groups were injected with either saline (1ml; 0.9%; i.p.) or IL-1β (10μl; 3.5ng/ml; i.c.v.), the third was injected i.c.v. with both IL-1β (3.5ng/ml) and SB 203580 (10μl; 50μM) prior to the induction of LTP. Test shocks were given at 30 sec intervals for a 10 min control period to tetanization. High frequency stimulation, consisting of 3 trains of stimuli of 250 Hz for 200 msec at 30 sec intervals, were delivered to the perforant path, as indicated by the arrow. LTP was induced in saline-treated rats, compared to IL-1 treated rats, whereby there was an inability to sustain LTP. This effect was reversed in rats treated with both IL-1β and SB 203580. The data presented here are the means of 6 observations for each group. The data is expressed as the mean percentage change in epsp slope with time.


300
200
100
0
EPSP Slope (%)
Saline
IL-1β
IL-1β+SB203508
0
10
20
30
40
TIME (min)

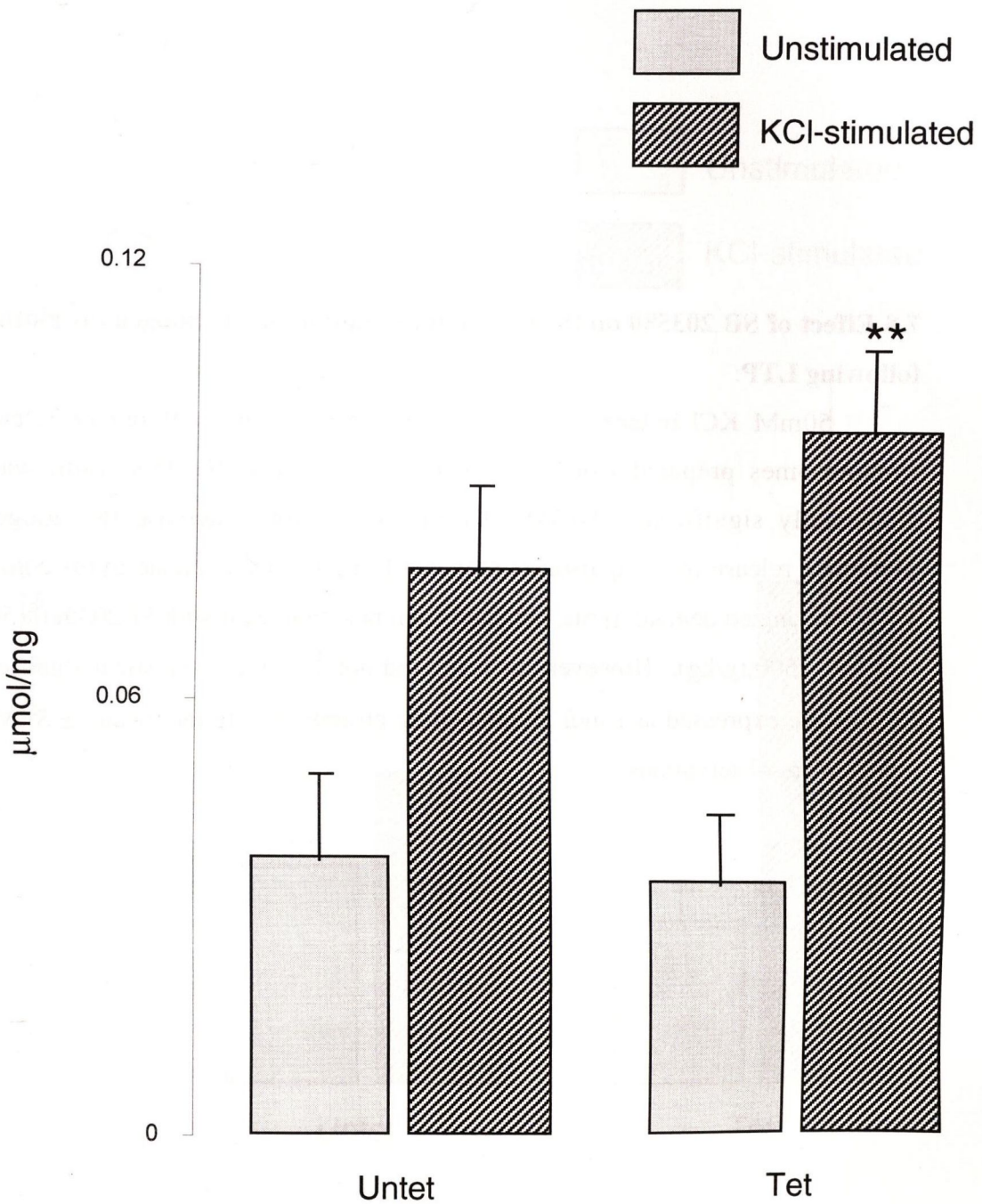
7.6 Effect of SB203580 on the LPS-induced impairment of LTP:

Three groups of Male Wistar rats were anaesthetized by i.p injection of urethane (1.5g/kg). Two groups were injected i.p. with either saline (1ml; 0.9%) or LPS (1ml; 500μg/kg), the third was injected with both LPS (1ml; 500μg/kg; i.p.) and SB203580 (10μl; 50μM; i.c.v.) 3 h prior to the induction of LTP. Test shocks were given at 30 sec intervals for a 10 min control period to tetanization. High frequency stimulation, consisting of 3 trains of stimuli of 250 Hz for 200 msec at 30 sec intervals, were delivered to the perforant path, as indicated by the arrow. LTP was induced in saline-treated rats, compared to LPS treated rats, whereby there was an inability to sustain LTP. This effect was reversed in rats treated with both LPS and SB203580. The data presented here are the means of 6 observations for each group. The data is expressed as the mean percentage change in epsp slope with time.


Saline
LPS
LPS+SB203508
300
200
100
0
Epsp Slope (%)
0
10
20
30
40
TIME (min)

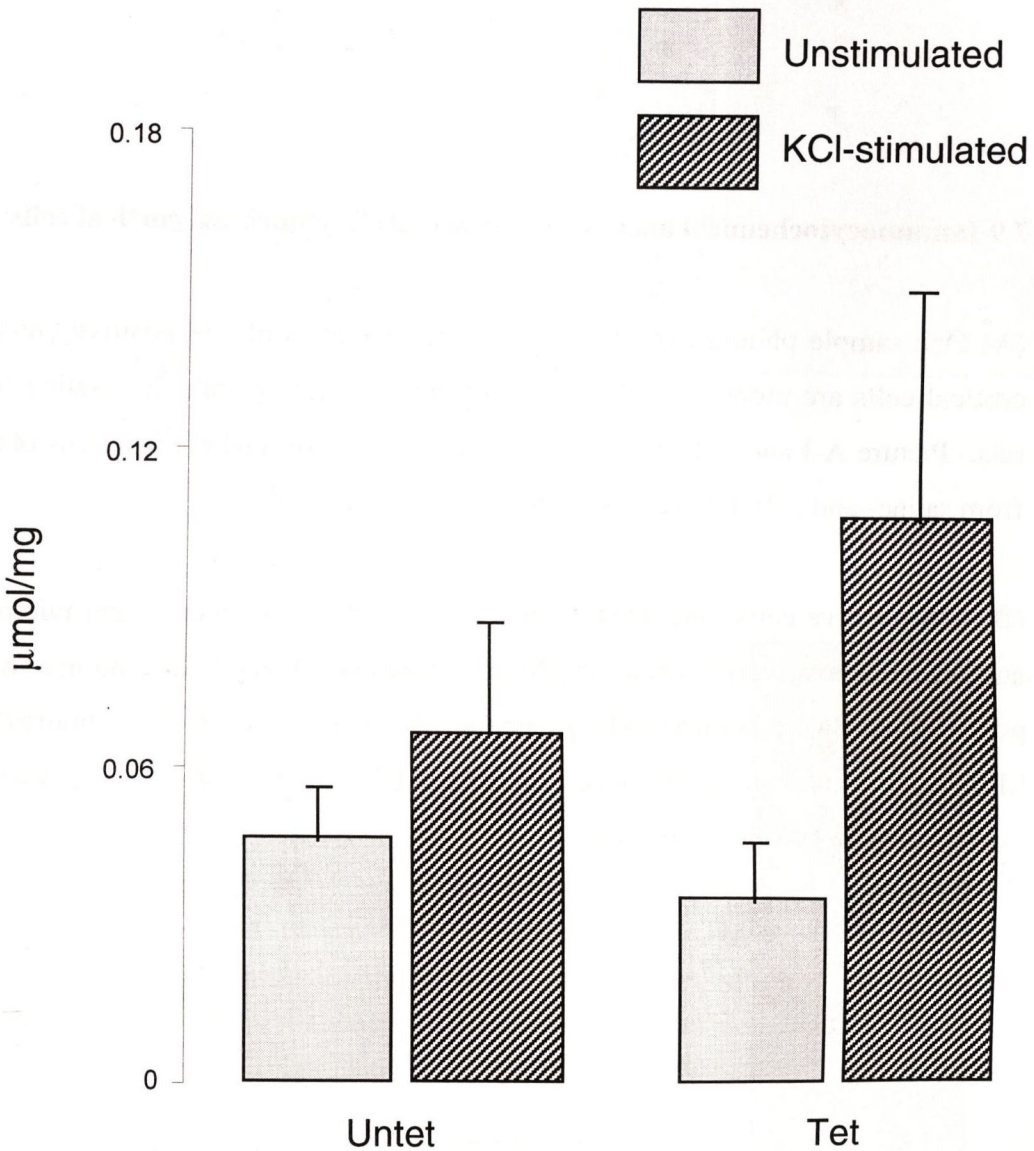
7.7 Effect of SB203580 and on the IL-1β-induced inhibition of glutamate release following LTP:

50mM KCl markedly increased endogenous glutamate release in synaptosomes prepared from unetanized dentate gyrus, but this result was not statistically significant. 50mM KCl caused a further significant increase in endogenous glutamate release in synaptosomes prepared from tetanized dentate gyrus compared with untetanized dentate gyrus, obtained from rats pre-treated with SB203580 (50μM) and IL-1β (3.5ng/ml: **$p<0.01$; Student's t-test). Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 6 independent observations.


Unstimulated
KCl-stimulated
0.12
0.06
0
µmol/mg
**
Untet
Tet

7.8 Effect of SB 203580 on the LPS-induced inhibition of endogenous glutamate following LTP:

50mM KCl induced a slight increase in endogenous glutamate release in synaptosomes prepared from unetanized dentate gyrus, but this result was not statistically significant. 50mM KCl caused a further increase in endogenous glutamate release in synaptosomes prepared from tetanized dentate gyrus compared with untetanized dentate gyrus, obtained from rats pretreated with SB203580 (50μM) and LPS (500μg/kg). However this result did not reveal any statistical significance. Results are expressed as μmol glutamate/mg protein and are the means ± SEM of 6 independent observations.

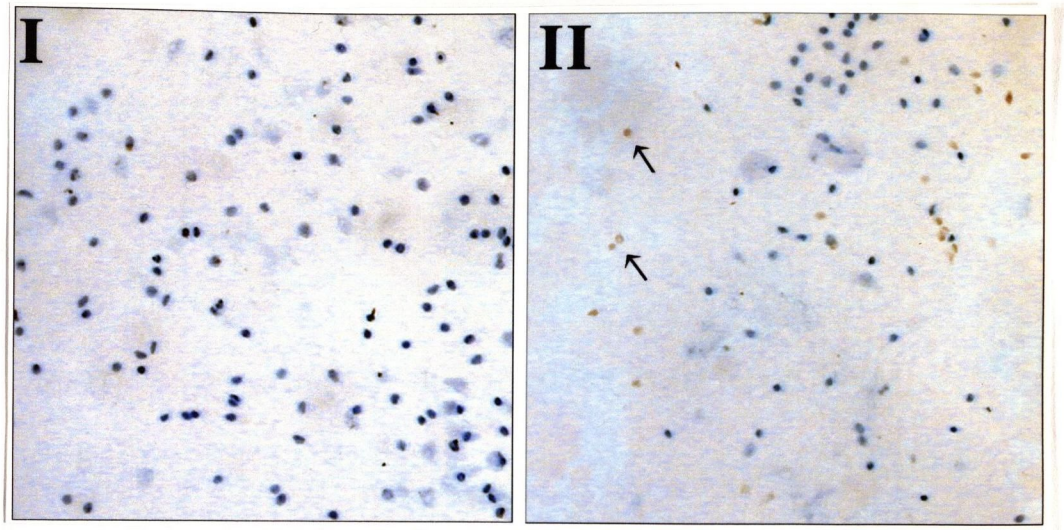

Unstimulated
KCl-stimulated
0.18
0.12
0.06
0
µmol/mg
Untet
Tet

7.9 Immunocytochemical analysis of p38 activity in entorhinal cortical cells:

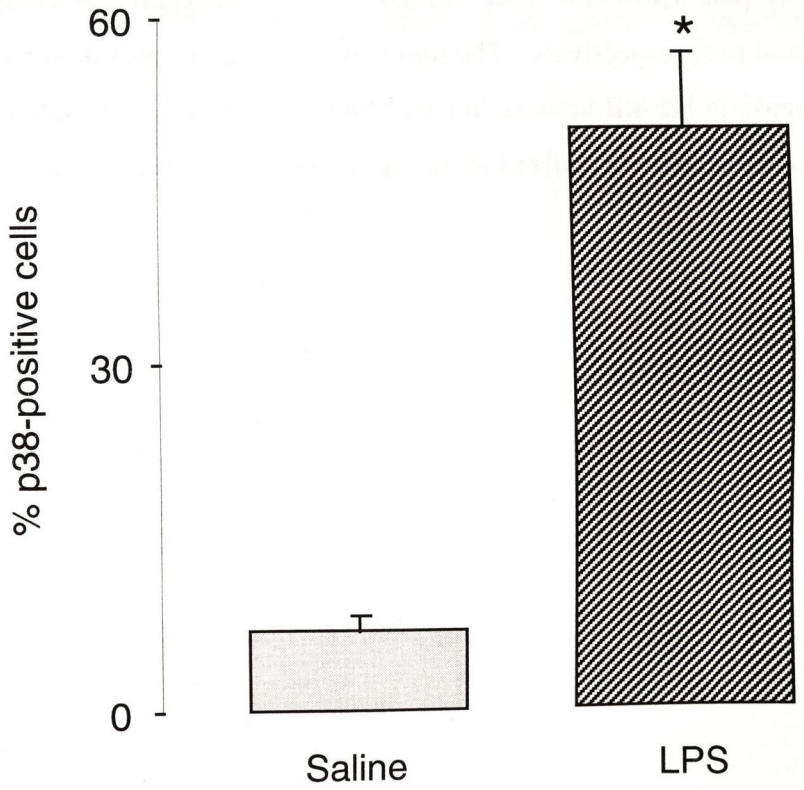
(A) One sample photograph illustrates that the numbers of p38-positive entorhinal cortical cells are more abundant in LPS-treated (500μg/kg) rats than saline-treated rats. Picture A-I and A-II illustrate p38-staining in entorhinal cortical cells obtained from saline- and LPS-treated rats, respectively.

(B) p38-positive cells (indicated by arrows) were counted under a light microscope and the cells positively stained for p38 were noted (x 40 mag.). The number of p38-positive cells were significantly greater in entorhinal cortical cells obtained from LPS-treated rats than saline-treated rats (*$p<0.05$; student's t-test). The results are expressed as % p38-positive cells.

(A)



(B)



7.10 Effect of LPS on the transcriptional activity of NF-κB:

One sample immunoblot is presented here, indicating that the transcriptional activity of NF-κB was markedly increased in entorhinal cortical nuclear extracts processed from LPS-treated (500μg/kg) rats compared to that of saline-treated rats. Every pair illustrated in the immunoblot is activity of NF-κB for saline- and LPS-treated rats, respectively. The immunoblot illustrates that 4 out of the 6 pairs show an increase in NF-κB activity in LPS-treated rats compared to saline-treated rats. These increases can be visualized in the pairs marked 1, 2, 5 and 6.

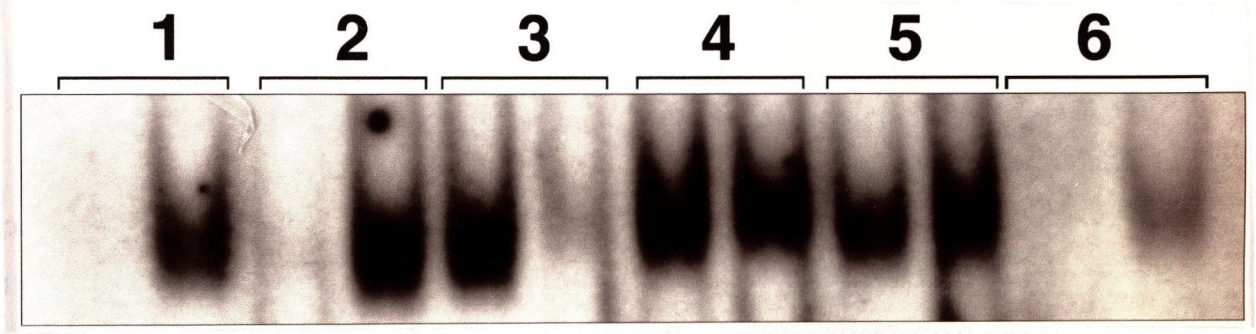

1
2
3
4
5
6

Fig 7.11 Analysis of the effect of LPS on caspase-3 activity:

Hippocampal slices from LPS-treated (500μg/kg) rats showed a significant increase in caspase-3 activity compared with that of slices from saline-treated rats (**p< 0.01; Student's t-test). Results are expressed as nmol AFC/mg protein/min and are the means ± SEM of 6 independent observations.

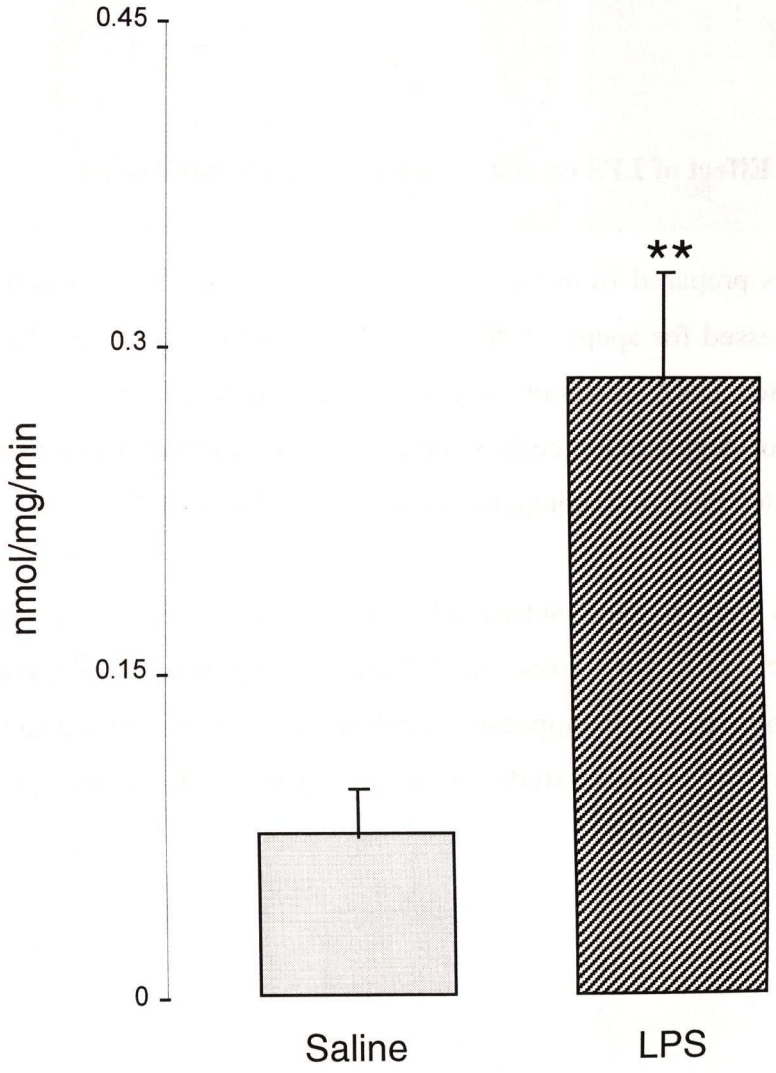
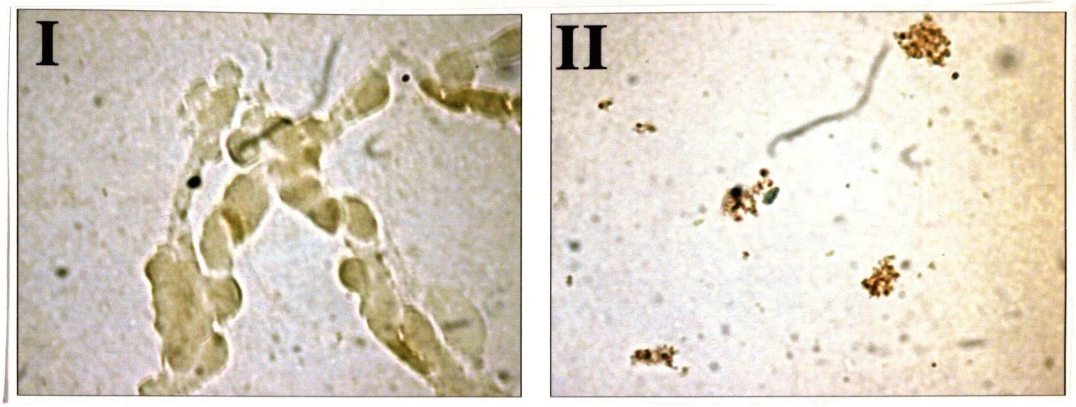

0.45
0.3
0.15
0
nmol/mg/min
**
Saline
LPS

Fig 7.12 Effect of LPS on cell viability in hippocampal cells:

(A) Cells prepared from hippocampus of saline- and LPS-treated (500μg/kg) rats were assessed for apoptotic death by TUNEL assay, a system which end-labels the fragmented DNA of apoptotic cells. One sample photograph illustrates that the number of degenerating cells is more abundant in hippocampal cells prepared from LPS-treated rats (A-II) compared to saline-treated rats (A-I).

(B) Cells were counted under a light microscope and the cells positively stained for apoptotic death were noted (x 100 mag). The number of apoptotic cells were significantly greater in hippocampal cells obtained from LPS-treated rats than saline-treated rats (**$p<0.01$; student's t-test). The results were expressed as the % degenerative cells.

(A)



(B)

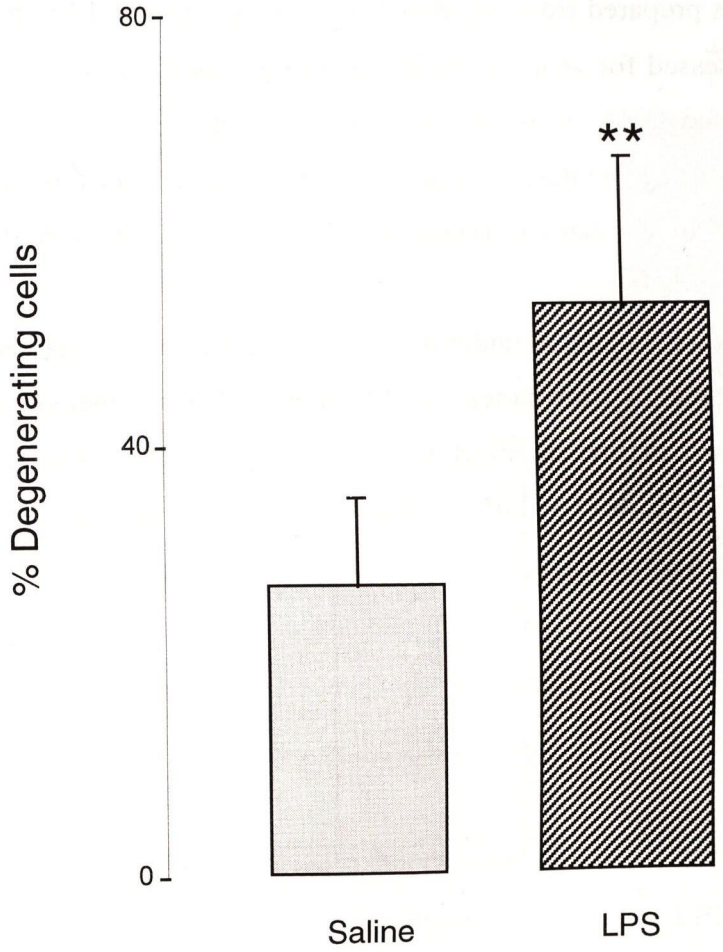
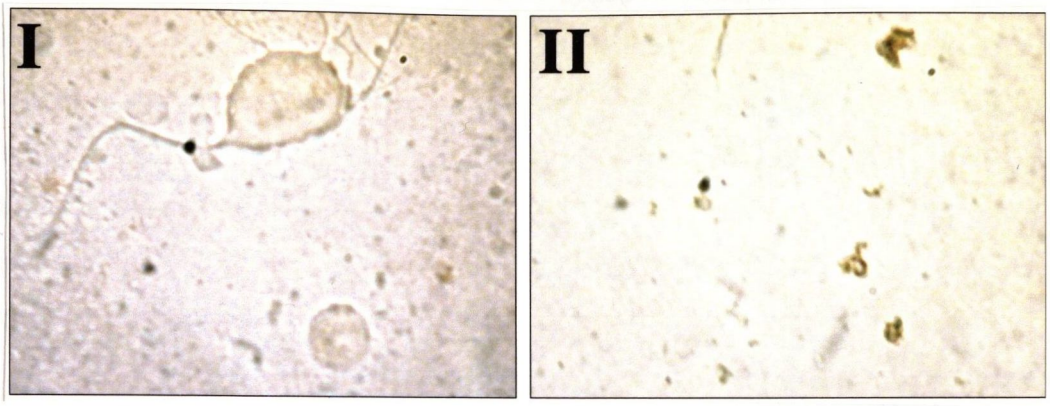


Fig 7.13 Effect of LPS on cell viability in entorhinal cortical cells:

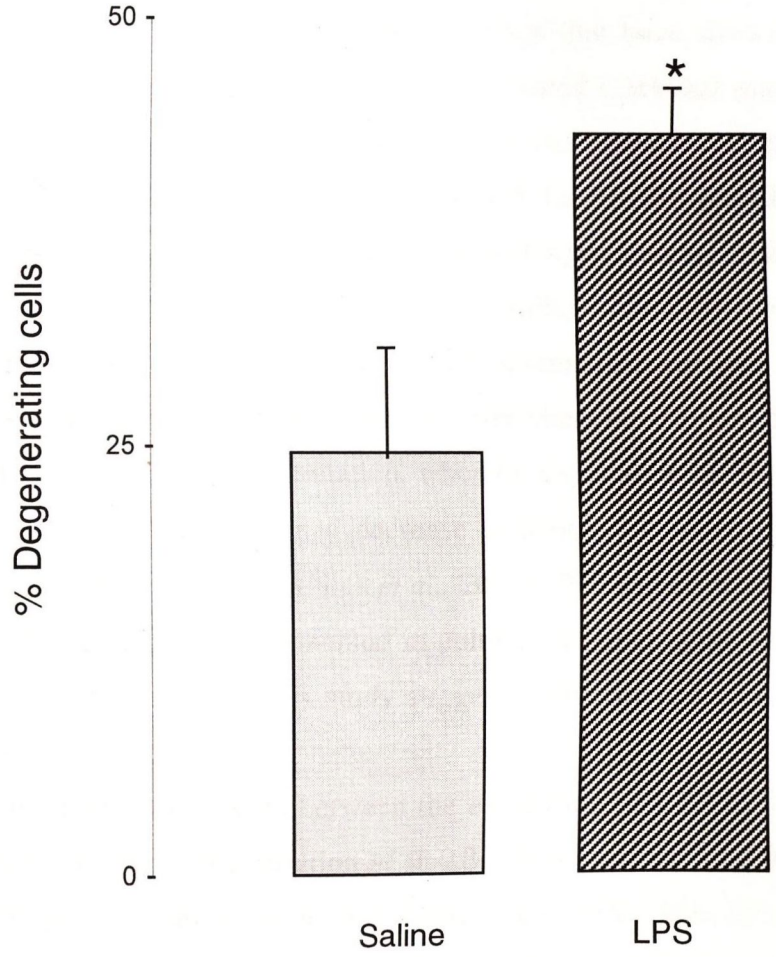
(A) Cells prepared from entorhinal cortex of saline- and LPS-treated (500μg/kg) rats were assessed for apoptotic death by TUNEL assay, a system which end labels the fragmented DNA of apoptotic cells. One sample photograph illustrates that the number of degenerating cells are more abundant in entorhinal cortical cells prepared from LPS-treated rats (A-II) compared to saline-treated rats (A-I).

(B) Cells were counted under a light microscope and the cells positively stained for apoptotic death were noted (x 100 mag.). The number of apoptotic cells were significantly greater in entorhinal cortical cells obtained from LPS-treated rats than saline-treated rats (*$p<0.05$; student's t-test). The results were expressed as the % degenerative cells.

(A)



(B)



7.4 Discussion:

In previous chapters it has been shown that increased IL-1β concentration is associated with impaired synaptic functions and is implicated in lipopolysaccharide (LPS) induced changes in synaptic function. Furthermore, the evidence suggested a role for stress activated protein kinases in IL-1β-induced impairments. The primary aim of this study was to further elucidate the extent of the damage induced by LPS on synaptic function. This study addresses two principal questions; firstly, to what extent does LPS administration effect neuronal viability and markers of neurodegeneration ? Secondly, what role might p38 play in this ?

The first series of experiments were undertaken to investigate the possibility for a role for p38 in LPS-induced impaired synaptic function. The effect of LPS-treatment on p38 activity in hippocampal tissue was assessed and the results indicated that p38 activity was significantly enhanced in hippocampal tissue following LPS-treatment. This result is consistent with other findings that have shown an LPS-induced increase in p38 activity. For example, LPS-treated C3H/OuJ macrophages mediated the phosphorylation of p38, with an optimal response after 30 min (Medvedev *et al.,* 2000). This result is comparable with the finding that LPS caused an increase in p38 activity in primary cultures of astrocytes and microglia (Bhat *et al.,* 1998). Similarly, exposure of human neutrophils to LPS resulted in increased p38 activity in a concentration-dependent manner and the optimal response was reached at 20-25 min (Nick *et al.,* 1996). The same group of researchers have reported a role for p38 in LPS-induced pulmonary inflammation, whereby application of SB 203580, a specific inhibitor of p38, resulted in a decrease in neutrophil function which is prominent in inflammatory responses (Nick *et al.,* 2000). Pyo and co-workers (1998) determined that p38 activity was augmented in cultured rat microglial cells. These reports and the data presented in this study suggest a role for p38 in response to bacterial infection.

A negative correlation exists between the expression of LTP in the dentate gyrus and an increase in the concentration of IL-1β. Moreover, the inhibitory effect of IL-1β on LTP in rat dentate gyrus *in vivo* (Murray & Lynch, 1998a,b) was shown to be accompanied by an inhibitory effect on glutamate release (Murray *et al.,* 1998). On the basis of these reports, the next series of experiments were undertaken to establish whether the LPS leads to an augmentation in IL-1β concentration, which

might negatively impact on the expression of LTP and the results indicate that IL-1β was increased in hippocampal tissue in LPS-injected rats. This result is in agreement with other groups that have reported a similar finding. LPS was reported to increase the concentration of IL-1β in various cell types; for example, in macrophages and glia (Loscher, personal communication, Kong *et al.,* 1997). Furthermore, LPS causes an increase in the concentration of IL-1β in rat meningeal macrophages (Van Dam *et al.,* 1998) and also IL-1β mRNA in meningeal cells (Quan *et al.,* 1999). These reports and the findings presented in this study, suggest that LPS may mediate its inhibitory effect on the expression of LTP through an increase in IL-1β concentration.

Many studies have begun to concentrate on the processes that are required for the activation of IL-1β, since it plays a central role in the pathogenesis of chronic and acute inflammatory diseases and in neurological disorders (Dinarello, 1996). The activation of IL-1β occurs by means of activation of the heterodimeric cysteine protease, ICE, which cleaves pro-IL-1β into a mature and active form (Thornberry *et al.*, 1992). To investigate how the LPS-induced increase in IL-1β transpired in the present study, ICE activity was assessed in hippocampal slices obtained from saline- and LPS-treated rats. Not surprisingly, an increase in ICE activity was observed in hippocampal slices obtained from LPS-treated rats, compared with saline-treated rats. The data are consistent with an earlier finding showing that ICE activity was increased in cultured monocytic and endothelial cells (Schumann *et al.,* 1998) and human monocyte-derived macrophages (Hilbi *et al.,* 1997), subsequent to LPS treatment. It is significant that the inhibition of ICE activity by a specific inhibitor, resulted in attenuation in IL-1β-associated ischemic and excitotoxic neuronal damage (Hara *et al.,* 1997). These reports and the data presented in this study, suggest that ICE activation plays a pivotal role in IL-1β-induced impairments. On the basis of the findings outlined in this section, it could be hypothesized that among the initial changes induced by LPS is an increase in ICE activity with the subsequent increase in IL-1β concentration. The data presented here propose that among the downstream consequences of these changes are, an increase in the activity of p38, a decrease in glutamate release and impaired LTP.

To further investigate a possible role for p38 in synaptic function, the next series of experiments involved the use of a selective p38 inhibitor, SB 203580. The effect of SB 203580 on the inhibitory effect of glutamate release was assessed in

dentate gyrus synaptosomes. This result has previously been described and discussed in chapter 3. Briefly, KCl-stimulated glutamate release was significantly increased in synaptosomes prepared from control dentate gyrus, but this effect was not seen in synaptosomes treated with IL-1β. Preincubation of synaptosomes with both SB 203580 and IL-1β resulted in restoration of the response to KCl stimulation. This result suggests that p38 activity plays a role in the IL-1β-inhibition of glutamate release.

Because p38 activity has been implicated in glutamate release *in vitro*, and because LTP in dentate gyrus synapses is associated with an increase in glutamate release, it was decided to assess whether this kinase may be implicated in the IL-1β- and LPS-inhibition of LTP. The effect of intracerebroventricular (i.c.v.) injection of SB 203580 was assessed in IL-1β- and LPS-treated rats. The present results indicate that IL-1β impairs the effect of LTP, as discussed previously in chapter 4. Furthermore, the results show that SB 203580 reversed the IL-1β-induced impairment in LTP, thus suggesting a role for p38 in the impairment of LTP. In this study a high very high dose of LPS (500μg/kg) was administered by intraperitoneal injection 3 hours prior to the induction of LTP and, under these experimental conditions, the data indicate that LPS impaired the expression of LTP. The immediate increase in the epsp slope following tetanic stimulation was attenuated, suggesting that both the induction and maintenance of LTP were inhibited by LPS. This result is identical to the result previously described and discussed in chapter 6, whereby LPS impaired LTP, although a lower concentration of LPS was administered. A previous study examined the effect of LPS (500μg/kg) on spatial memory recall in the Morris water maze. The results showed that LPS (500 μg/kg) had no effect on learning ability, therefore suggesting that, although the dose is relatively high, it has no effect on spatial learning (Brooks, *pers. com.*).

The results show that SB203580 reversed the LPS-induced impairment in LTP. It is evident that SB203580 further enhanced the expression of LTP in the rat dentate gyrus following LPS-treatment, but there is no obvious explanation for this. These results are consistent with the finding that SB 203580 attenuates the IL-1β-induced inhibition of LTP and NMDA-EPSPs in the rat dentate gyrus *in vitro* (Coogan *et al.,* 1999b). This result suggests that IL-1β inhibits LTP by activating p38. This report and the data presented in the present study indicate that p38 maybe

involved in the signalling mechanisms by which IL-1β and LPS inhibit LTP, both *in vivo* and *in vitro*. In this study the effect of SB203580 alone was not assessed, but it has been previously reported by Coogan *et al* (1999b) that hippocampal slices pretreated with SB203580 did not lead to any significant changes in NMDA-receptor mediated transmission. This data suggests that the inhibitor alone has no effect on synaptic function.

As discussed in previous chapters, LTP is associated with a concomitant increase in glutamate release from perforant path granule cell synapses (Bliss & Collingridge, 1993; McGahon & Lynch, 1996; McGahon *et al.,* 1997), and therefore endogenous glutamate release was assessed in this study. Initially, the effect of saline and LPS pretreatment on glutamate release was assessed in dentate gyrus synaptosomes. LPS treatment caused a significant decrease in KCl-stimulated endogenous glutamate release, whereas glutamate release assessed in dentate gyrus synaptosomes obtained from saline-treated rats was increased upon KCl-depolarization. These findings suggest that the LPS-induced impairment in LTP also affect the biochemical changes leading to an increase in glutamate release in LTP. To address the hypothesis that p38 plays an inhibitory role in synaptic function, the effect of i.c.v. injection of SB 203580 was assessed on endogenous glutamate release in untetanized and tetanized dentate gyrus synaptosomes prepared from IL-1β- and LPS-treated rats. The results indicate that SB 203580 reversed the IL-1β-induced inhibitory effect on glutamate release. Similarly, SB 203580 reversed the LPS-induced inhibition of glutamate release, but not to the same extent that the IL-1β-induced inhibition was reversed, suggesting a close coupling between these variables as previously reported (McGahon & Lynch, 1996; O'Donnell *et al.,* 2000). It might even be suggested that the LPS-induced inhibition of glutamate release is responsible for the impairment in LTP. From these findings it may also be proposed that LPS-mediates its inhibitory effects by increasing IL-1β concentration in the hippocampus, and thereby enhancing activation of the p38 signalling pathway.

The second part of this study aimed to explore the extent of LPS-induced neurodegeneration. One of the most profound effects that are associated with neurodegeneration is the activation of the cell death or apoptotic pathway. Regulation of caspase activation is indicative of apoptotic cell death. Caspase-3 is one of the key mediators of apoptosis and is responsible for the cleavage of many proteins associated

with the cell death pathway (Nicholson *et al.,* 1995). Since caspase-3 activity appears to be fundamental to apoptotic cell death, the effect of LPS administration on caspase-3 activity was assessed in rat hippocampal tissue and the results indicate that the activity of caspase-3 is increased in hippocampal tissue obtained from LPS-treated rats. This result is consistent with other studies that have shown that caspase-3 activity is increased in certain cell types, when exposed to certain apoptotic stimuli. For example, caspase-3 activity was enhanced in rat superior cervical ganglion neurons (SCGs) when subjected to NGF-withdrawal and staurosporine-induced apoptosis (McCarthy *et al.,* 1997). The role of caspase-3 was confirmed by the use of inhibitors. Ac-DEVD-CHO, a specific inhibitor of caspase-3, reversed NGF-withdrawal-induced apoptosis in SCG neurons (McCarthy *et al.*, 1997). Reportedly, LPS-induced apoptosis was accompanied by an increase in caspase-3 activity in Kupffer cells (Hamada *et al.,* 1999) and septo-hippocampal cell cultures following mechanical stretch injury (Pike *et al.,* 2000). In addition, it has been shown that Benzo(a)pyrene, a potent procarcinogen and toxicant, induced apoptotic cell death in a Hepa1c17 hepatoma cell line and that this was associated with activation of caspase-3 (Lei *et al.,* 1998).

One effective way to assess apoptotic cell death is by visualizing morphological changes that are associated with cell death. Typical features that characterize cell death include DNA fragmentation, chromatin condensation, mitochondrial damage and formation of apoptotic bodies (Thornberry & Lazebnik, 1998). In this study, hippocampal and entorhinal cortical dissociated cells prepared from saline- and LPS-treated rats, were assessed by TUNEL (TdT-mediated dUTP Nick-End Labelling) which functions by end-labelling the fragmented DNA of apoptotic cells. The results indicate that the percentage of TUNEL-positive cells was significantly greater in hippocampal and entorhinal cortical cells obtained from LPS-treated rats compared with saline-treated rats. This result is consistent with other reports that have shown an increase in degenerative cells following physiological stress or insult. For example, LPS injection induced a time- and dose-dependent increase in the number of TUNEL-positive hepaptocyte cells obtained from rats (Hamada *et al.,* 1999). Brain tissue samples removed from adults following traumatic brain injury showed an increase in TUNEL-positive neurons and glial cells (Clark *et al.,* 1999). Similarly, in sections obtained from mice with traumatic spinal cord

injury, neuronal apoptotic cell death was evident as the percentage TUNEL-positive cells were significantly greater than in control mice (Li *et al.,* 2000). These findings indicate that physiological stress, including LPS treatment, induces degeneration and suggests that neuronal cell death in the hippocampus and entorhinal cortex is a consequence of LPS injection as assessed by TUNEL staining and increased caspase-3 activity.

More recent studies have turned to investigating the role transcription factors play in cell signalling in apoptosis. The transcription factor, nuclear factor-kappa B (NF-κB) is activated in response to cytokines, neurotrophic factors, and viral and bacterial infections (Baeuerle, 1998). Activation of NF-κB appears to be crucial for glial and neuronal cell function, particularly under stressful conditions (O'Neil & Kaltschmidt, 1997) and appears to play a pivotal role in apoptotic events (Martin *et al.,* 1999). However, certain results have been interpreted as indicating an anti-apoptotic role for NF-κB. To further investigate the effects of peripheral administration of LPS on cell signalling pathways, the transcriptional activity of NF-κB was assessed in nuclear extracts obtained from dissociated entorhinal cortical cells harvested from LPS-treated rats. The result indicated that LPS was associated with an increase in the transcription of NF-κB in entorhinal cortical cells. Although LPS-induced an increase in NF-κB activation, it stills remains to be elucidated whether the increase observed here is protective or degenerative. It could be hypothesised that the LPS-induced increase in NF-κB activity occurs as a protective measure in this situation, and the protective role of NF-κB has been reported previously. For example, Manna and co-workers (1999) showed that NF-κB activation was increased eight-fold in LPS-treated U-937 cells. From these studies it was also proposed that the LPS-induced activation of NF-κB was necessary to block TNF-induced apoptosis, and therefore NF-κB activation may act in an anti-apoptotic manner. NF-κB has been shown to play an anti-apoptotic role in cell death, where it has been reported that inhibition of NF-κB induced apoptosis in PC 12 cells, the effect of which is not prevented by treatment with nerve growth factor (Taglialatela *et al.,* 1997). In support of this finding, it has also been shown that sympathetic neurons undergo apopotosis when NF-κB activation is inhibited (Maggirwar *et al.,* 1998). Although NF-κB activation can prevent apoptosis in neuronal and non-neuronal cells (Mattson *et al.,* 2000), there is evidence that NF-κB plays a pro-apoptotic role in cell death in

particular circumstances. Reportedly, NF-κB inhibition reduces the extent of quinolinic-acid induced degeneration of rat striatal neurons (Qin *et al.,* 1998) and subsequently suppressed the quinolinic-acid induced expression of the pro-apoptotic gens, p53 and c-myc (Qin *et al.,* 1999). Generally, the vast majority of NF-κB-inducing agents are deleterious to cells and in response NF-κB targets many anti-and pro-apoptotic genes (Pahl, 1999).

The results presented in this study, suggest a possible link between the LPS-induced increase in NF-κB activation and p38 activity. Reportedly, p38 activity is necessary for the translocation and activation of NF-κB in myocardial adaptation to ischemia (Maulik *et al.*, 1998) and thus the activation of NF-κB was inhibited by SB 203580, a specific inhibitor of p38. Here, the activity of p38 as assessed by investigating the percentage of p38-positive stained cells using an antibody specific for p38, showed an LP-S induced increase in staining in entorhinal cortical cells. A role for p38 in apoptosis has been previously described but it seems that the role p38 is dependent on cell-type and is stimulus-dependent (Ono & Han, 2000). For example, trophic factor withdrawal-induced cell death in PC12 cells and rat fibroblasts results in apoptotic cell death, which is coupled with an increase in p38 activity (Kummer *et al.,* 1997). The same group demonstrated that inhibition of p38 reverses these effects. MacKay and Mochly-Rosen (1999) showed that ischemia-induced apoptosis in rat cardiac myocytes was down regulated upon application of the p38 inhibitor, SB 203580, again suggesting that p38 acts in a pro-apoptotic manner. Furthermore, it was shown that when PC12 cells were subjected to NGF-withdrawal, the subsequent apoptosis was accompanied by greatly enhanced p38 activity (Xia *et al.,* 1995). These reports, and the results presented in this study, suggest a role for p38 in neuronal cells death which may be linked with NF-κB activation as it has been recently showed that p38 is necessary for the activation of NF-κB (Vanden Berge *et al.*, 1998; Carter *et al.,* 1999).

The findings presented in this study suggest that the deleterious effects of LPS in the hippocampus are evident in synaptic function and neuronal viability, and are mediated by the activity of p38. The results revealed that intraperitoneal administration of LPS impaired the expression of LTP and the concomitant increase in glutamate release. The evidence suggests that the LPS-induced changes are mediated by an increase in ICE activity and thereby an increase in the concentration

of IL-1β, which then acts downstream to activate the p38 kinase. It is proposed that p38 is a key player in these impaired synaptic functions since the inhibitor of p38 blocked the LPS- and IL-1β-induced inhibition of LTP and glutamate release. The data show that an increase in NF-κB activation paralleled the increase in p38 activity. While these changes were coupled with evidence that cells of the entorhinal cortex and hippocampus were apoptotic, it remains to be established whether the LPS-induced activation of p38 and/or NF-κB are directly responsible for these changes.

VII Final Discussion

The principal aim of this study was to investigate the role of IL-1β in synaptic function in the rat hippocampus and the possible mechanisms involved in IL-1β-induced changes. To address the hypothesis that IL-1β affects synaptic transmission and synaptic plasticity in the hippocampus, the most appropriate model was considered to be long-term potentiation (LTP), a putative model for learning and memory (Bliss & Lømo, 1973). The induction of LTP in the dentate gyrus is accompanied by several biochemical changes and among them is an increase in glutamate release (McGahon & Lynch. 1996); this was the foundation upon which many of these studies were carried out.

Preliminary studies showed, firstly that IL-1β exerts an inhibitory effect on endogenous glutamate release in rat hippocampal and dentate gyrus synaptosomes *in vitro*. Secondly, accompanying this change was an increase in the activation of the stress-activated protein kinases, JNK and p38, giving rise to the concept that IL-1β may mediate its inhibitory effects through activation of JNK and p38. Thirdly, confirmation that increased JNK and p38 activation plays a role in mediating IL-1β-induced inhibitory effects was shown by the data obtained from the tissue of animals which had received intracerebroventricular (i.c.v.) injections of IL-1β prior to the induction of LTP *in vivo*. The data indicated that IL-1β impaired the expression of LTP and that this was coupled with decreased KCl-stimulated release of endogenous glutamate, concomitant with an increase in the activity of both JNK and p38. Fourthly, feeding rats an anti-oxidant diet enriched in vitamin E and vitamin C inhibited the IL-1β-induced effects, therefore, it is proposed that these effects are a consequence of an increase in IL-1β-induced increase in reactive oxygen species (ROS) formation. To corroborate these findings, a series of studies were carried out to investigate whether or not the IL-1β-induced inhibitory effects were similar in aged rats, as an age-related increase in IL-1β has previously been documented (Murray & Lynch, 1998a). The findings revealed that age was associated with an impairment in the expression of LTP and a parallel increase in the IL-1β concentration and activity of JNK and p38. The concept that ROS may be a critical mediator in these age-related changes was supported by the finding that these age-related changes were reversed by dietary manipulation.

IL-1β has been implicated in responses to infection and is thought to mediate changes associated with inflammation. Therefore, lipopolysaccharide (LPS), a known inducer of IL-1β (Ilyin *et al.,* 1998), was administered intraperitoneally (i.p.) to assess LPS-induced IL-1β changes in the hippocampus. The findings indicated that LPS impaired LTP and the concomitant increase in endogenous glutamate release, and that these changes were coupled with an increase in JNK and p38 activity and an increase in ROS formation. Increases in IL-1β concentrations and interleukin-1β-converting enzyme (ICE) was also observed, suggesting that the LPS-induced increase in IL-1β was brought about by an increase in ICE activity; this was confirmed by the finding that the ICE inhibitor, Ac-YVAD-CMK, reversed the LPS-induced changes. The final section of this thesis concentrated on examining the effect of LPS on synaptic function by specifically investigating neuronal viability and to establish whether increased activity of p38 plays a pivotal role in LPS-induced changes.

The data indicate that the inhibitory effect of LPS on LTP and the associated increase in glutamate release was mediated through activation of p38, as the p38 inhibitor, SB203580, reversed these effects. The data also show that an increase in p38 activity was in parallel with an increase in NF-κB activity. Apoptotic changes in cells of the entorhinal cortex and hippocampus coupled with an increase in p38 activity, suggested that p38 plays a key role in the LPS-induced impairments in synaptic function and neuronal viability.

The evidence presented here suggests an inhibitory role for IL-1β in synaptic functions and that these effects are mediated by an increase in the activity of the stress-activated proteins, JNK and p38. It has been previously reported by several groups that IL-1β exerts an inhibitory effect on the expression of LTP in CA1 (Bellinger *et al.,* 1993) CA3 (Katsuki *et al.,* 1990) *in vitro* and in rat dentate gyrus *in vivo* (Cunningham *et al.,* 1996; Murray & Lynch, 1998a,b). It has also been shown that, associated with the expression of LTP, is a concomitant increase in glutamate release in the rat dentate gyrus (Bliss *et al.,* 1986; McGahon *et al.,* 1996) and that IL-1β inhibits endogenous glutamate release in the rat hippocampus *in vitro* (Murray et al., 1997). These findings suggested that the inhibitory effect of IL-1β on the expression of LTP might be due to its inhibitory effect on release. It has been shown that IL-1β stimulates JNK and p38 activity in

various cell types, *in vitro*. The results of this study show that IL-1β increases JNK and p38 activity in synaptosomes prepared from rat hippocampus and dentate gyrus, the importance of this being that their activity may impact greatly on endogenous glutamate release. The concentrations of IL-1β used in these studies and in the experiments described in this thesis, are similar to pathophysiological concentrations (0.1-10ng/ml) found in *post mortem* tissue and cereobrospinal fluid of patients with chronic neurodegenerative disorders such as Alzheimer's disease (Rothwell *et al.,* 1998).

Once it was apparent that JNK and p38 activity was increased upon IL-1β stimulation in synaptosomes *in vitro*, the mechanisms involved in the IL-1β-, age-, and LPS-associated inhibition of LTP were assessed. Common to all three situations, was an increase in IL-1β concentration in hippocampus, and an increase in JNK and p38 activity and an increase in ROS formation, coupled with a decrease in endogenous glutamate release, in the rat dentate gyrus. Previous studies have reported that the age-related impairment in LTP in rat dentate gyrus *in vivo* was associated with an increase in the concentration of IL-1β and ROS formation (Murray & Lynch, 1998a,b) and decreased glutamate release (McGahon *et al.,* 1999). Furthermore, it was shown that there was an increase IL-1β concentration and ROS formation in dentate gyrus following an i.c.v. injection of IL-1β (O' Donnell *et al.,* 2000) and that these changes were coupled with impaired LTP.

The data from this study are the first to indicate that LTP in rat dentate gyrus is impaired following i.p. injection of LPS. Previous reports have indicated that injection of pertussis toxin, a potent exotoxin, inhibits tetanically-induced LTP in rat CA1 striatum hippocampal slices 3-4 days following injection (Goh & Pennefather, 1989). Similarly, pertussis toxin inhibited LTP in mossy-fiber CA3 pyramidal slices, but not in CA1 pyramidal slices (Ito *et al.,* 1988). Although these studies were carried out using a different protocol, the results are in broad agreement with the ones presented here. It was shown that IL-1β concentration was increased in the rat dentate gyrus following LPS injection. This is consistent with many other reports indicating that LPS is a potent inducer of IL-1β (Henderson *et al.,* 1996), in murine macrophages (Loscher, *pers. comm.*) and murine glial cell cultures (Kong *et al.,* 1997). It then follows that the LPS-induced increase in IL-1β is probably responsible for its inhibitory effect on the

expression of LTP and also accounts for the decrease in KCl-stimulated release of endogenous glutamate.

Although these results suggest that the mechanism by which IL-1β exerts its actions is through an increase in the activity of JNK and p38, the question as to how IL-1β concentration is initially increased in the hippocampus of aged, IL-1β-, and LPS-treated rats, remains to be addressed. It has been known for some time that in order for IL-1β to become activated, it must be firstly cleaved by a cysteine protease, namely, the interleukin-1β-converting enzyme (ICE; Thornberry *et al.,* 1992). The data presented here indicates that ICE activity increases concurrently with IL-1β production in the hippocampus of LPS-treated rats. This is in agreement with other findings which have demonstrated that LPS increases ICE activity in cultured monocytic and endothelial cells (Schumann *et al.,* 1998) and human monocyte-derived macrophages (Hilbi *et al.,* 1997). It is significant that the inhibition of ICE activity by a specific inhibitor resulted in an attenuation in IL-1β-induced ischemia and excitotoxic neuronal damage in the rat and mouse brain (Hara *et al.,* 1997) and the LPS-induced induction of IL-1β in the plasma and peritoneal fluid of mice, *in vivo* and *in vitro* (Fletcher *et al.,* 1995). From these reports and the findings presented in this study, it could be hypothesized that IL-1β is increased in aged- and LPS-treated rats through an increase in the activity of ICE. However, the actual cause of this increase in ICE activity remains to be addressed, although it has been suggested that ROS plays a pivotal role in caspase activation (Hampton *et al.,* 1998).

Common to all the three experimental circumstances discussed here (age, IL-1β treatment and LPS-treatment) is a positive correlation between an increase in the formation of ROS and IL-1β concentration. The data reported here whereby, associated with an increase in IL-1β in IL-1β- and LPS-treated rats, is an increase in the formation of ROS in hippocampal synaptosomes, is consistent with the previous findings that ROS formation is increased in aged rats *in vivo* (O' Donnell *et al.,* 2000). However, *in vitro* studies have illustrated that, not only can IL-1β induce an increase in ROS formation in rat hippocampal tissue, but hydrogen peroxide induced an increase in the concentration of IL-1β in hippocampal tissue, indicating that a possible positive feedback mechanism is involved. It could be speculated that IL-1β production increases the formation of ROS,

which creates a positive impact on IL-1β production. To test the hypothesis that ROS formation is a causative link between an increase in IL-1β concentration and impaired synaptic function, an antioxidant diet, enriched in vitamin E and vitamin C was fed to saline- and IL-1β-treated rats. Dietary manipulation largely reversed the IL-1β-induced and age-related inhibition of LTP and glutamate release in the rat dentate gyrus. From these studies it could be proposed that ROS is a key player in the IL-1β-induced inhibition of synaptic function whereby both act in concert. It has recently been shown that bovine chondrocytes stimulated with IL-1β, revealed an increase in the activity of IL-1β-induced JNK activity and that anti-oxidant treatment with ascorbic acid and *N*-acetylcysteine reversed these effects (Lo *et al.,* 1996). These results, and the data presented in this study, suggest that ROS may act by increasing the concentration of IL-1β, the effect of which may positively impact on ROS formation, giving rise to an increase in the activity of JNK, or indeed p38, although p38 was not examined by Lo and co-workers (1996).

The present results suggest that increased IL-1β concentration is the underlying cause of the IL-1β-, age- and LPS-associated inhibition of LTP and these IL-1β-induced effects are mediated through an increase in the activity of JNK and p38. Because there is no commercially available inhibitor of JNK, the possible role of JNK could not be explored. However, the positive correlation between JNK activation and IL-1β concentration and the negative correlation between these two measures and impaired LTP, suggest that JNK is a likely candidate for mediating the IL-1β-induced effects. The availability of the p38 inhibitor, SB203580, made it to be feasible to explore the role p38 plays in these IL-1β-induced inhibitory effects. The data indicated that SB203580 co-applied with high (100ng/ml) and low (10pg/ml) concentrations of IL-1β reversed the IL-1β-induced inhibition of endogenous glutamate release in the rat hippocampus and dentate gyrus in *vitro,* respectively. Similarly, i.c.v. injection with SB230580 in IL-1β- and LPS-treated rats, prior to the induction of LTP in the rat dentate gyrus *in vivo*, attenuated the IL-1β- and LPS-induced inhibition of LTP. It was also shown that SB203580 markedly attenuated the IL-1β- and LPS-induced inhibition of glutamate release. These results provide compelling evidence that p38 plays a role in mediating these effects.

It remains unclear as to how p38 induces its inhibitory effects. It must be predicted that p38 acts on one or more substrates involved in both the expression of LTP and release of endogenous glutamate. One possibility is that p38 acts by inhibiting NMDA-receptor function. It has recently been shown that the IL-1β-induced inhibition of LTP and NMDA receptor-mediated synaptic transmission in the rat dentate gyrus *in vitro* (Coogan *et al.,* 1999a) is reversed by the application of SB203580. Not only is this finding in agreement with the data presented here, but it also suggests that p38 exerts its inhibitory effects on the NMDA-receptors, thus depressing its function, leading to an inhibition of LTP. The same group also showed the long-term potentiation of the synaptic response induced by using the potassium channel blocker, tetraethylammonium (TEA) was inhibited by application of IL-1β and that SB 203580 blocked this effect (Coogan *et al.,* 1999b). Thus it could be speculated that p38 may mediate its effects by activating potassium channels; therefore a possible substrate for p38 activity might be one subunit of the potassium channel. A further explanation for the proposed p38-induced inhibition of glutamate release, is the possibility that calcium channel currents are compromized by increased activity of p38. It has been previously reported that an increase in IL-1β exerts an inhibitory effect on calcium channel currents in hipocampal neurons of adult guinea pigs (Plata-Salamán & Ffrench-Mullen, 1994) and cultured cortical neurons (MacManus *et al.,* 2000). Furthermore, IL-1β inhibits calcium influx in rat hippocampal synaptosomes *in vitro* (Murray *et al.,* 1997). It could be hypothesized that these IL-1β-induced inhibitory effects are mediated by an increase in p38 and/or JNK activity, which might then lead to impaired glutamate release. One of the most common mechanisms by which elevated intracellular calcium regulates cellular events is through its association with calmodulin; for example, Mullany *et al* (1996) demonstrated that an increase in endogenous glutamate release in hippocampal synaptosomes is accompanied by an increase in calcium/calmodulin-dependent kinase II (Ca/CaMKII). One of the substrates for Ca/CaMKII is the synaptic vesicle protein synapsin, which plays a role in tethering synaptic vesicles into a cytoskeletal meshwork (Greengard *et al.,* 1993) preventing docking and fusion. Phosphorylation of synapsin by CaCaMKII interferes with the coupling of synapsin and actin, thus liberating vesicles from the cytoskeletal meshwork, thus enabling the vesicles to dock at the presynaptic plasma membrane

(Lynch, 1998). Additionally, it has been shown that phosphorylation of synaptophysin, which functions by forming a pore fusion, is concomitantly increased with the expression of LTP (Mullany & Lynch, 1997). In relation to this study, it could be theorized that p38 and/or JNK exert their actions by inhibiting protein-protein interactions that are necessary for transmitter release. It is possible that one or more of the proteins of the synaptic vesicle membrane or synaptic plasma membrane may be a substrate for p38 and/or JNK.

In this study, the LPS-induced increase in IL-1β was accompanied by an increase in the activity of caspase-3 in hippocampal slices, a potent marker for cell death and it was shown that the activity of the transcription factor, NF-κB, was coupled with an increase in p38 activity as well as an increase in p38-positively stained entorhinal cortical cells. Additionally, dissociated entorhinal cortical and hippocampal cells prepared from LPS-treated rats, revealed an increase in the number of apoptotic cells. Previous reports have shown in tissue prepared from ischemic rats that p38 activity is necessary for the translocation and activity of NF-κB (Maulik *et al.,* 1998) and similarly in monocytes (Carter *et al.,* 1999); SB 203580 inhibited NF-κB activation in both cases. It is of interest here that a synergistic role for Caspase-3 and p38 has been demonstrated in calyculin-induced cell death in cortical neurons (Ko *et al.,* 2000).

It seems reasonable to propose that the LPS-induced inhibition of LTP may be linked with cell death in the rat dentate gyrus and entorhinal cortical cells, both of which showed evidence of undergoing apoptosis in parallel with an increase p38 activity. It is important to note that the integrity of the cells of the entorhinal cortex is essential for the expression of LTP since the perforant path originates in the entorhinal cortex and projects to the granule cells of the dentate gyrus (Bliss & Lynch, 1988). It could be suggested that if entorhinal cortical cells have undergone apoptosis, then the integrity of the perforant path will be affected and that stimulation of the perforant-path may not be sufficient to allow the expression of LTP. Mullany and Lynch (1997) have shown that, associated with the expression of LTP in the rat dentate gyrus, is a concurrent increase in protein synthesis in the entorhinal cortex. This effect is impaired in aged rats. There is compelling evidence here to suggest that hippocampal and entorhinal cortical apoptotic cell death plays a contributing role in the LPS-induced inhibition of LTP. Recent evidence has shown that β-amyloid (Mattson *et al.,* 1998b), staurosporine and iron

(Mattson et al., 1998a) induce synaptic apoptosis in cortical synaptosomes, whereby typical markers of apoptosis were activated, namely, caspase activity and nuclear changes. Although the concept that cell death has been only discussed here in relation to LPS-induced inhibition of LTP, these changes may also hold for the IL-1β- and age-related inhibition of LTP since recent evidence suggests that apoptosis is evident in entorhinal cortical cells, prepared from aged rats (Brady *et al, pers. comm.*).

The findings of this study suggest that the cascade of events that underlies the inhibition of LTP and glutamate release is mediated through an increase in the concentration of IL-1β, the effects of which are mediated though an increase in the activity of p38 and/or JNK. It is known that the actions of IL-1β are receptor-mediated, such that when IL-1 binds to IL-1R1, the IL-1 receptor accessory protein (IL-1RAcP) forms a complex with IL-1RI and then recruits IRAK to the receptor complex. An adapter protein, MyD88, facilitates this action by docking to the complex, then phosphorylates IRAK, which consequently activates TRAF6 (as discussed in chapter 1). It could be hypothesized that this is the cascade of events which initiates the inhibitory effects of JNK and p38 in aged and IL-1β-treated rats, leading to impairments in synaptic function. However, it must be considered that the LPS-induced effects are initiated via its receptor, TLR4, which by some unknown manner activates IRAK (Swantek *et al.*, 2000).

Fig VII.I illustrates the proposed mechanism by which IL-1β-, age- and LPS induced increase in IL-1β exerts its effects, i.e through the activation of JNK and p38, both of which may negatively impact on synaptic function, either by (1) inhibiting the function of synaptic vesicle proteins involved in the release of endogenous glutamate, or (2) through the induction of apoptotic effects. These effects are detrimental to the functional efficacy of the hippocampus, the consequence of which is inhibition of the expression of LTP and the concomitant increase in glutamate release.

Future Work:

The findings presented in this study suggest a role for JNK and p38 in impaired synaptic function, their effect being initiated by an increase in the concentration of IL-1β. A number of observations described provide a possible starting point for several further studies. For example:

1) Determination of possible substrates of p38 and JNK which might impact on glutamate release:

   Does increased activity of p38 and JNK modulate calcium influx? i.e. are calcium channel subunit proteins substrates for JNK and/or p38?

   Does p38 and JNK play a role in potassium and calcium channel activity?

   Does p38 and JNK modulate the interaction between the synaptic plasma membrane and synaptic vesicle proteins?

2) Examination of the possibility that p38 and JNK may induce apoptotic effects in dentate gyrus and entorhinal cortex in aged rats and IL-1β-treated rats:

   Assessment of the evidence that "synaptic apoptosis" exists (Mattson *et al.*, 1998a).

3) A further investigation into the mechanism involved in the activation of p38 and JNK

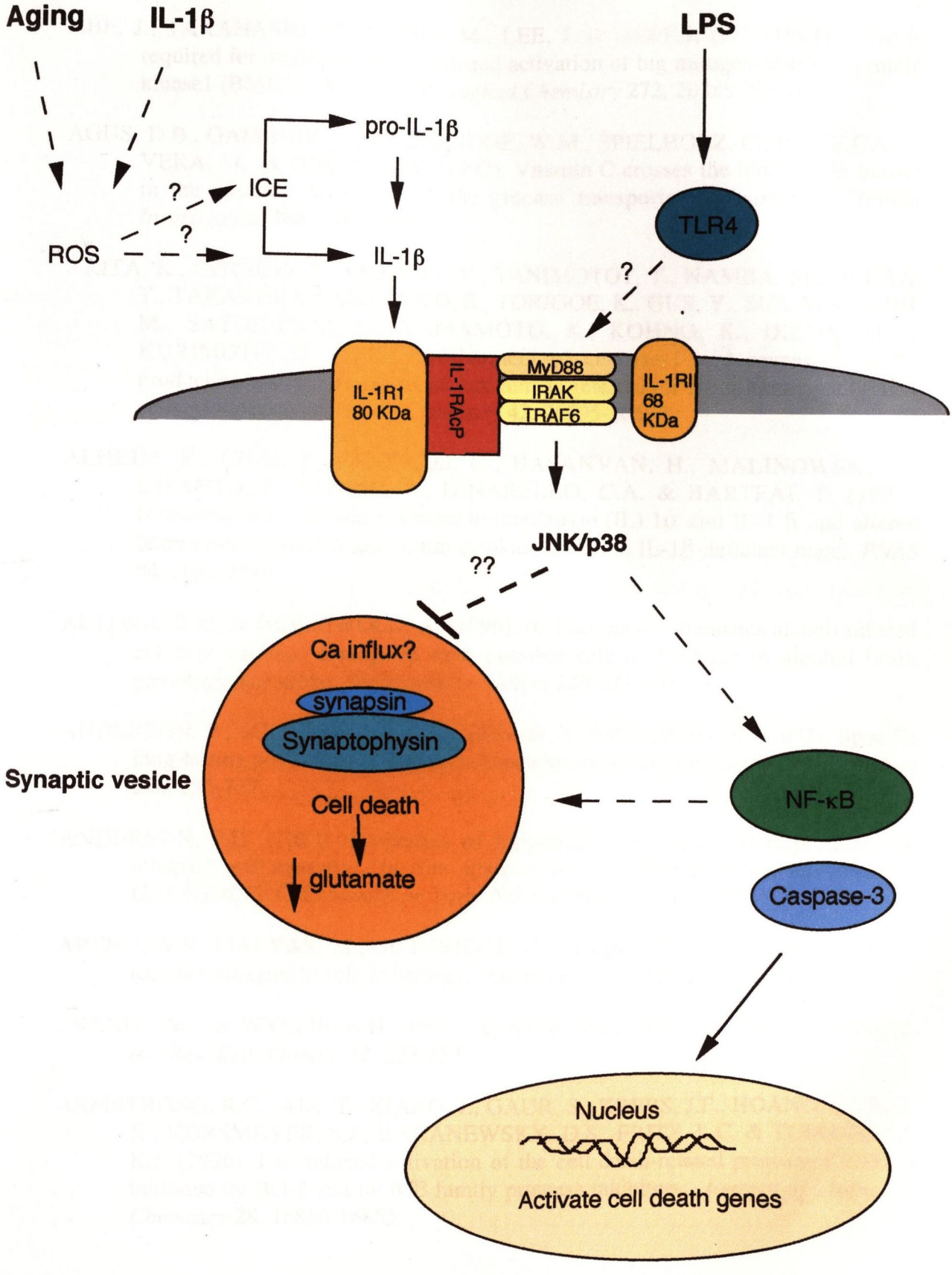
   What precise role does IRAK play in the induction of p38 and JNK?

   What other transcription factors are activated concurrently with p38 and JNK under stressful circumstances? AP-1? ATF-2?

   Is there a role for these transcription factors at a synaptic level?

Fig. VII.I Schematic representation of the proposed signalling pathway:

ICE cleaves pro-IL-1β to IL-1β, following possible activation of ROS formation through an IL-1β- and age-related increase in IL-1β. The activity of IL-1β is receptor mediated. IL-1β proceeds to activate JNK and p38, which in turn may negatively impact on synaptic protein vesicles resulting in impaired glutamate release and expression of LTP. The mechanism by which LPS exerts its actions is also receptor mediated, but differs from that of the IL-1β signalling pathway. LPS binds to TLR4, which, through the phosphorylation of IRAK, activates p38, and JNK. The activation of JNK and p38 results in an increase in NF-κB and caspase-3 activity, which may also impair glutamate release possibly by apoptotic cell death. Thus, this schematic proposes that firstly, the increased activity of p38 and/or JNK may cause inhibition of glutamate release via possible inhibition of synaptic vesicle proteins. Secondly, p38 and/or JNK activity may play a pivotal role in initiating apoptotic cell death, by activating pro-apoptotic gene transcription. However, the sequence of events that may be involved in this process remains to be fully elucidated.


Aging
IL-1β
LPS
pro-IL-1β
ICE
?
ROS
?
IL-1β
TLR4
?
IL-1R1
80 KDa
IL-1RAcP
MyD88
IRAK
TRAF6
IL-1RI
68
KDa
JNK/p38
??
Ca influx?
synapsin
Synaptophysin
Synaptic vesicle
Cell death
glutamate
NF-κB
Caspase-3
Nucleus
Activate cell death genes

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X Appendices

Appendix I
Solutions used

Krebs solution

NaCl, 136mM

KCl, 2.54mM

KH_2PO_4, 1.18mM

$MgSO_4.7H_2O$, 1.18mM

$NaHCO_3$, 16mM

Glucose, 10mM

Containing $CaCl_2$, 2mM

Separating gel

Acrylamide/Bisacrylamide (30% stock) 33% v/v

Tris-HCl, 1.5M, Ph 8.8

SDS, 1% w/v

Ammonium persulphate, 0.5% w/v

Distilled water

TEMED, 0.1% v/v

Stacking gel

Acrylamide/Bisacrylamide (30% stock) 6.5% v/v

Tris-HCl, 0.5M, pH 6.8

SDS, 1% w/v

Ammonium persulphate, 0.5% w/v

Distilled water

TEMED, 0.1% v/v

Electrode running buffer

Tris base, 25mM

Glycine, 200mM

SDS, 17mM

Phosphate-buffered saline (PBS), pH 7.4

Na_2HPO_4, 80mM

NaH_2PO_4, 20mM

NaCl, 100mM

PBS-Tween (PBS-T)

0.1% Tween-20 solution in PBS.

Transfer buffer, pH 8.3

Tris base, 25mM

Glycine, 192mM

Methanol, 20% v/v

SDS, 0.05% w/v

Distilled water

Sample buffer

Tris-HCl, 0.5M, pH6.8

Glycerol, 10% v/v

SDS, 0.05% w/v

β-mercaptoethanol, 5% v/v

Bromophenol blue, 0.05% w/v

Preparation of acrylamide

Acrylamide (29.2g) was added to N'N'Bis-methylene-acrylamide (0.8g) and dissolved in 100ml of distilled water. This solution was filtered and stored in the dark at 4oC for a maximum of one month.

Lysis Burfer pH 7.4:

HEPES, 25mM

$MgCl_2$, 5mM

DTT, 5mM

EDTA, 5mM

PMSF, 2mM

Leupeptin, 10µg/ml

Pepstatin, 10µg/ml

Incubation Buffer:

HEPES, 100mM

DTT, 5mM

PBS pH 7.4-Dissociated cells:

NaCl, 137mM

Na_2HPO_4, 8.1mM

KH_2PO_4, 1.47mM
KCL. 2.68mM

Buffer A pH 7.9:

HEPES, 10mM
$MgCl_2$, 1.5mM
KCL, 10mM
PMSF, 0.5mM
DTT, 0.5mM

Buffer C pH 7.9

HEPES, 0.5mM
NaCl, 20mM
$MgCl_2$, 1.5mM
EDTA, 0.2mM
PMSF, 0.5mM
Glycerol, 25% v/v

Buffer D pH 7.9

HEPES, 10mM
KCl, 50mM
EDTA, 0.2mM
PMSF, 0.5mM
Glycerol, 20% v/v

Appendix II

Suppliers	**Address**
Affiniti	Affiniti Research Products Ltd., Mamhead Castle, Mamahead, Exeter EX6 8HD, U.K.
Amersham	Amersham International plc, Lincoln Place, Green End, Aylesbury, Buckinghamshire HP20 2TP, U.K.
Beeline Healthcare	Beeline Healthcare Ltd., Clondalkin Road, Clondalkin, Dublin 24.
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hampstead, Hertfordshire, HP2 7TD, U.K.
Calbiochem	Calbiochem/Novabiochem, European Distribution Centre, Boulevard Industrial Park, Nottingham NG9 1BR, U.K.
Genzyme Diagnostics	Genzyme Diagonistics, 1 Kendall Square, Cambridge, MA 2139, U.S.A.
Lennox	Lennox Laboratory Supplies, John F. Kennedy Drive, Naas Road,

	Dublin 12, Ireland.
Molecular Probes	Molecular Probes Europe B.V., PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.
Promega	Promega Corporation, 2800 Woods Hollow Rd., Madison, Wisconsin, U.S.A.
Santa Cruz	Santa Cruz Biotechnology, Inc., 2161 Delaware Avenue, Santa Cruz, C.A. 95060 U.S.A.
Sartorius	Sartorius, 5 Sussex Street, Dun Laoghaire, Co. Dublin ,Ireland.
Sigma	Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K.
Vector	Vector Laboratories, 16, Wulfric Square, Peterborough PE3 8RF, U.K.
Whatman	Whatman International Ltd., Maidstone, Kent, U.K.

XI List of publications

1. **Vereker, E.**, Campbell, V., Roche, E., McEntee, E. and Lynch, M.A. (2000) Lipopolysaccharide inhibits long-term potentiation in the rat dentate gyrus by activating caspase-1. *J. Biol. Chem.* **275** 26252-26258

2. **Vereker, E.**, O'Donnell, E. and Lynch, M.A. (2000) The inhibitory effects of interleukin-1β on LTP is coupled with increased activity of stress-activated kinases. *J. Neurosci.* **20** (18), 6811-68119

3. O'Donnell E., **Vereker, E.** and Lynch, M.A. (2000) Age-related impairment in LTP is accompanied by enhanced activity of stress-activated protein kinases: Analysis of underlying mechanisms. *Eur. J.Neurosci.* **12** (1) 345-352.

4. Whittaker, E., **Vereker, E.** and Lynch, M.A. (1999) Neuropeptide Y inhibits glutamate release and long-term potentiation in rat dentate gyrus. *Brain Research* **827** 229-233

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Lipopolysaccharide Inhibits Long Term Potentiation in the Rat Dentate Gyrus by Activating Caspase-1*

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Lipopolysaccharide, a component of the cell wall of Gram-negative bacteria, may be responsible for at least some of the pathophysiological sequelae of bacterial infections, probably by inducing an increase in interleukin-1β (IL-1β) concentration. We report that intraperitoneal injection of lipopolysaccharide increased hippocampal caspase-1 activity and IL-1β concentration; these changes were associated with increased activity of the stress-activated kinase c-Jun NH_2-terminal kinase, decreased glutamate release, and impaired long term potentiation. The degenerative changes in hippocampus and entorhinal cortical neurones were consistent with apoptosis because translocation of cytochrome *c* and poly(ADP-ribose) polymerase cleavage were increased. Inhibition of caspase-1 blocked these changes, suggesting that IL-1β mediated the lipopolysaccharide-induced changes.

There is increasing awareness of the existence of bidirectional communication between the immune and nervous systems. The proinflammatory cytokine, interleukin-1β (IL-1β),[1] is one molecule that may play a pivotal role in integrating neuronal immune responses with those of the endocrine system because it exerts significant effects in all systems, for example in response to stressors such as infection. Gram-negative bacterial infections are associated with multiple pathophysiological changes; it is widely accepted that these changes are stimulated by lipopolysaccharide (LPS), a component of the outer membrane of most Gram-negative bacteria. These changes, which include fever, changes in sleep pattern, and anorexia (1), are mimicked by, and therefore thought to be mediated through production of, IL-1β. Thus LPS, injected centrally or peripherally, increases IL-1β concentrations (2, 3) and IL-1β mRNA expression (4) in rat brain.

Although it appears that in certain circumstances IL-1β may be neuroprotective, the consensus is that prolonged exposure, or exposure of tissue to high concentrations of IL-1β, results in degenerative changes (5). Therefore it is significant that increased IL-1 concentrations in different brain areas have been correlated with neurodegenerative disorders such as Down syndrome, Alzheimer's disease (6), and Parkinson's disease (7), whereas in experimental models, IL-1β is considered to be responsible for the cell damage associated with ischemia (8) and excitotoxicity (9) and is increased after experimental traumatic lesions (10). A striking example of a neuronal deficit induced by IL-1β is the impairment in long term potentiation (LTP) in the hippocampus *in vitro* (11–13) and *in vivo* (14–16).

IL-1β is produced by glia (17, 18) and neurones (19, 20) in response to tissue stress. It is cleaved from the inactive percursor, pro-IL-1β, by the action of caspase-1, a member of a large family of cysteine proteases that have been implicated in apoptotic cell death (21–25). It might be predicted therefore that any trigger such as LPS, which induces an increase in IL-1β, will do so by increasing activity of caspase-1.

Our objective was to investigate the cellular consequences of an increase in IL-1β concentration in hippocampus in an effort to establish the mechanism by which IL-1β inhibits LTP in dentate gyrus. Intraperitoneal injection of LPS stimulated caspase-1 activity and induced an increase in IL-1β concentration, and these changes were paralleled by an increase in activity of the stress-activated protein kinase c-Jun NH_2-terminal kinase (JNK), a decrease in glutamate release, and inhibition of LTP in perforant path granule cell synapses. These changes, and the degenerative changes in neurones of the hippocampus and entorhinal cortex, were reversed by caspase-1 inhibition.

EXPERIMENTAL PROCEDURES

Induction of LTP in Vivo—Six groups of six male Wistar rats (250–350 g), obtained from the BioResources Unit, Trinity College Dublin, were anesthetized by intraperitoneal injection of urethane (1.5 g/kg). All rats groups received 1 ml of saline or 1 ml of LPS (200 μg/kg) intraperitoneally; four groups were pretreated either with an intracerebroventricular injection of 5 μl of saline or 5 μl of the caspase-1 inhibitor (10 pmol of Ac-YVAD-CMK, 2.5 mm posterior to Bregma, 0.2 mm lateral to midline, 3.5-mm depth) prior to the intraperitoneal injection and monitored for 3 h. A bipolar stimulating electrode and a unipolar recording electrode were placed in the perforant path (4.4 mm lateral to Lambda) and in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to Bregma), respectively, and 0.033-Hz test shocks were given for 10 min before, and 40 min after, tetanic stimulation (three trains of stimuli delivered at 30-s intervals, 250 Hz for 200 ms (26)). Rats were killed by cervical dislocation; cross-chopped slices (350 × 350 μm) were prepared from ipsilateral and contralateral dentate gyri, entorhinal cortex, and hippocampus and used to prepare dissociated cells (see below) or frozen separately in Krebs solution containing 10% dimethyl sulfoxide (27) and stored at −80 °C. For analysis, slices were thawed rapidly and rinsed in fresh oxygenated Krebs solution before preparation of homogenate or the crude synaptosomal pellet P_2 (26).

Analysis of Reactive Oxygen Species Formation—Formation of reactive oxygen species was assessed by measuring 2′7′-dichlorofluorescein (DCF), the oxidized, fluorescent product of 2′7′-dichlorofluorescein diacetate (DCFH-DA (28)). Synaptosomes prepared from hippocampal slices were incubated at 37 °C for 15 min in the presence of 10 μl of 5 μM DCFH-DA (from a stock of 500 μM) in methanol and centrifuged at

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[1] The abbreviations used are: IL-1β, interleukin-1β; LPS, lipopolysaccharide; LTP, long term potentiation; JNK, c-Jun NH_2-terminal kinase; DCF, 2′7′-dichlorofluorescein; 2′7′-dichlorofluorescein diacetate; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; ANOVA, analysis of variance; epsp, excitatory post-synaptic potential.

13,000 × g for 8 min at 4 °C to yield pellets that were resuspended in 2 ml of ice-cold 40 mM Tris buffer, pH 7.4, and monitored for fluorescence at 37 °C (excitation, 488 nm; emission, 525 nm).

Analysis of Caspase-1 Activity—Cleavage of the caspase-1 substrate (YVAD peptide, Alexis Corporation) to its fluorescent product was used as a measure of caspase-1 activity. Slices of tissue were washed, homogenized in 400 μl of lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, pH 7.4), subjected to four freeze-thaw cycles, and centrifuged at 15,000 rpm for 20 min at 4 °C. 90-μl samples of supernatant were added to 10 μl of 500 μM YVAD peptide and incubated at 37 °C for 60 min. 900 μl of incubation buffer (100 mM HEPES containing 10 mM dithiothreitol, pH 7.4) was added, and fluorescence was assessed (excitation, 400 nm; emission, 505 nm).

Analysis of IL-1β Concentration—IL-1β concentration in homogenate prepared from hippocampus of entorhinal cortex was analyzed by enzyme-linked immunosorbent assay (14; Genzyme Diagnostics). Antibody-coated (2.0 μg/ml final concentration, diluted in 0.1 M sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse IL-1β antibody) 96-well plates were incubated overnight at 4 °C, washed several times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, blocked for 2 h at 37 °C with 250 μl of blocking buffer (0.1 M PBS, pH 7.3, with 4% bovine serum albumin), and incubated with 50-μl IL-1β standards (0–1,000 pg/ml) for 1 h at 37 °C. Samples were incubated with 100 μl of secondary antibody (final concentration 0.8 μg/ml in PBS containing 0.05% Tween 20 and 1% bovine serum albumin; biotinylated polyclonal rabbit anti-mouse IL-1β antibody) for 1 h at 37 °C, washed, and incubated in 100 μl of detection agent (horseradish peroxidase-conjugated streptavidin; 1:1,000 dilution in PBS containing 0.05% Tween 20 and 1% bovine serum albumin) for 15 min at 37 °C. 100 μl of tetramethylbenzidine (Sigma) was added, incubated at room temperature for 10 min, and absorbance read at 450 nm within 30 min.

Analysis of JNK Phosphorylation, Cytochrome c Translocation, and Poly(ADP)-ribose Polymerase (PARP) Cleavage—JNK phosphorylation was analyzed in samples prepared from hippocampal tissue; cytochrome *c* translocation and PARP cleavage were analyzed in samples prepared from entorhinal cortex. In the case of JNK and PARP, tissue homogenates were diluted to equalize for protein concentration (29), and 10-μl aliquots (1 mg/ml) were added to 10 μl of sample buffer (0.5 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.05% bromphenol blue, w/v), boiled for 5 min, and loaded onto gels (10% SDS for PARP and 12% for JNK). In the case of cytochrome *c*, the cytosolic fraction was prepared by homogenizing slices of entorhinal cortex in lysis buffer (composition in mM: 20 HEPES, pH 7.4, 10 KCl, 1.5 $MgCl_2$, 1 EGTA, 1 EDTA, 1 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aprotonin), incubating for 20 min on ice, and centrifuging (15,000 × *g* for 10 min at 4 °C). The supernatant (*i.e.* cytosolic fraction) was suspended in sample buffer (150 mM Tris-HCl, pH 6.8, 10% glycerol v/v, 4% SDS w/v, 5% β-mercaptoethanol v/v, 0.002% bromphenol blue w/v) to a final concentration of 300 μg/ml, boiled for 3 min, and loaded (6 μg/lane) onto 12% gels. In all cases proteins were separated by application of a 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min), and immunoblotted with the appropriate antibody. To assess JNK activity, proteins were immunoblotted with an antibody that specifically targets phosphorylated JNK (Santa Cruz Biotechnology, Inc.; 1:2,000 in PBS and 0.1% Tween 20 containing 2% non-fat dried milk) for 2 h at room temperature. Immunoreactive bands were detected using peroxidase-conjugated anti-mouse IgG (Sigma) and enhanced chemiluminescence (Amersham Pharmacia Biotech). To assess cleavage of PARP, we immunoblotted with an antibody (1:2,000) raised against the epitope corresponding to amino acids 764–1014 of PARP of human origin (Santa Cruz Biotechnology Inc.), and immunoreactive bands were detected using peroxidase-conjugated anti-rabbit IgG (Sigma) and enhanced chemiluminescence. To assess cytochrome *c*, a rabbit polyclonal antibody raised against recombinant protein corresponding to amino acids 1–104 of cytochrome *c* (Santa Cruz Biotechnology Inc.) was used. Immunoreactive bands were detected using peroxidase-conjugated anti-rabbit antibody (Sigma) and enhanced chemiluminescence.

Release of Glutamate—Synaptosomal tissue prepared from untetanized and tetanized dentate gyrus was resuspended in ice-cold Krebs solution containing 2 mM $CaCl_2$, aliquotted onto 0.45-μm Millipore filters, and rinsed under vacuum. Tissue was incubated in 250 μl of oxygenated Krebs solution ± 40 mM KCl at 37 °C for 3 min, and the filtrate was collected and stored. To analyze glutamate concentration, triplicate 50-μl samples or 50-μl glutamate standards (50 nM to 10 μM in 100 mM Na_2HPO_4 buffer, pH 8.0) were added to 320-μl glutaraldehyde (0.5% in 100 mM NaH_2PO_4 buffer, pH 4.5)-coated 96-well plates and incubated for 60 min at 37 °C (30). 250 μl of ethanolamine (0.1 M in 100 mM Na_2HPO_4 buffer) and 200 μl of donkey serum (3% in PBS-T) were used to bind unreacted aldehydes and to block nonspecific binding, respectively. Samples were incubated overnight at 4 °C in the presence of 100 μl of anti-glutamate antibody (raised in rabbit, 1:5,000 in PBS-T, Sigma), washed with PBS-T, and then incubated for 60 min at room temperature with 100 μl of anti-rabbit horseradish peroxidase-linked secondary antibody (1:10,000 in PBS-T, Amersham Pharmacia Biotech). 100 μl of 3,3′,5,5′-tetramethylbenzidine liquid substrate was added, incubation continued for exactly 60 min, 30 μl of 4 M H_2SO_4 was added to stop the reaction, and optical densities were determined at 450 nm.

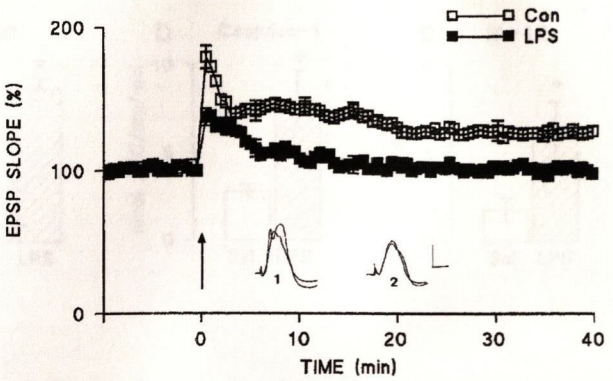

FIG. 1. **Intraperitoneal injection of LPS inhibits LTP in perforant path-granule cell synapses.** The mean population epsp slope immediately after tetanic stimulation was attenuated in LPS-treated rats compared with saline-treated rats and was close to base line at the end of the 40-min recording period. The data are means of six individual experiments, and S.E. values are included for every 10th response. Sample recordings in the 5 min immediately before tetanic stimulation and in the last 5 min of the experiment are superimposed for saline-injected (*trace 1*) and LPS-injected (*trace 2*) rats. The *scale bars* represent 1 mV and 2 ms.

Dissociation of Cells and Analysis of Cell Viability—350-μm slices prepared from entorhinal cortex and hippocampus were equilibrated in oxygenated Krebs solution for 30 min at 30 °C and then incubated in Krebs solution containing 1 mg/ml protease X, 1 mg/ml protease XIV, and 1,600 Kunitz Dnase for 30 min at 30 °C. Washed slices were resuspended in 1 ml of prewarmed Dulbecco's modified essential medium containing 1,600 Kunitz Dnase, triturated with a glass Pasteur pipette, and passed through a nylon mesh filter to remove tissue clumps. 30-μl aliquots were plated out on poly-L-lysine-coated 11-mm round glass coverslips, placed in a 5% CO_2 incubator at 37 °C for 1 h, and fixed in 4% paraformaldehyde (30 min at room temperature). Coverslips were stored at 4 °C in PBS until use (31).

Cells were stained using the Rapi-diff II staining procedure (DiaCheM International Ltd., Lancastershire, U. K,) and viewed under × 100 magnification. Cells displaying degenerative features (*e.g.* shrinkage and membrane blebbing) were counted and expressed as a percentage of the total number of cells examined (80–100/coverslip in the case of entorhinal cortex and 100–200 in the case of the hippocampus).

Statistical Analysis—A one-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), *post hoc* Student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other. Student's *t* test was used to establish statistical significance in some cases; for example, when analysis was performed on tissue prepared from untetanized and tetanized tissue obtained from the same rat.

RESULTS

LPS Blocks LTP by Increasing IL-1β Concentration in the Hippocampus and Activating JNK—Tetanic stimulation delivered to the perforant path 3 h after intraperitoneal injection of LPS resulted in an increase in the mean slope of the population epsp; the mean percentage increase in the 2 min immediately following tetanic stimulation (± S.E., compared with the 5 min immediately before tetanic stimulation) was 133.58 (± 3.48),

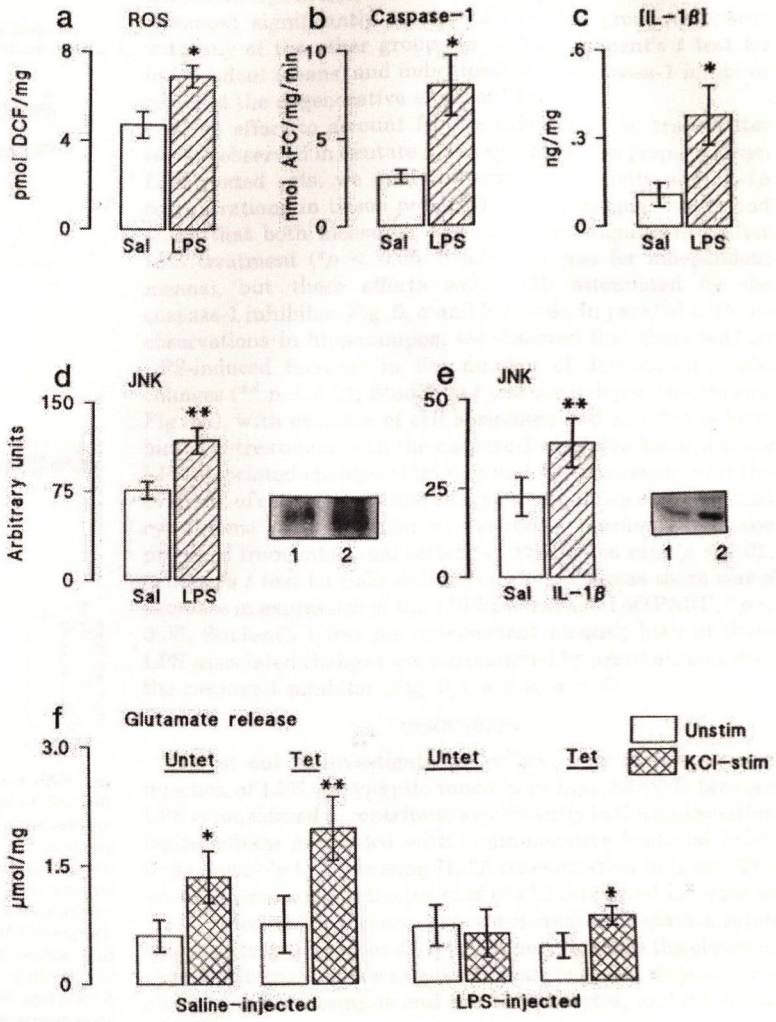

FIG. 2. **The LPS-stimulated increases in caspase-1 activity and IL-1β concentration are accompanied by changes in JNK activity and glutamate release.** Intraperitoneal injection of LPS induced significant increases in reactive oxygen species (*ROS*) production (*panel a*), caspase-1 activity (*panel b*), IL-1β concentration (*panel c*), and JNK activity (*panel d*) in hippocampal tissue (*$p < 0.05$, **$p < 0.01$, Student's t test for independent means, $n = 6$), whereas IL-1β (1 ng/ml) significantly increased activity of JNK *in vitro* (*panel e*, * $p < 0.05$, Student's t test for paired values, $n = 6$). Sample immunoblots indicate the stimulatory effects of LPS injection and IL-1β *in vitro* (*lanes 2* in panels *d* and *e*, respectively) compared with the corresponding controls. The addition of 40 mM KCl to synaptosomes prepared from untetanized dentate gyrus of saline-injected rats significantly increased glutamate release (*$p < 0.05$, ANOVA, *panel f*), but this effect was enhanced in synaptosomes prepared from tetanized dentate gyrus (**$p < 0.01$, ANOVA). The addition of KCl to synaptosomes prepared from untetanized dentate gyrus of LPS-injected rats failed to enhance release, whereas the response was attenuated in synaptosomes prepared from tetanized tissue.

but this was not maintained so that the mean percentage increase in population epsp slope in the last 5 min of the experiment was 100.81 ± 2.26. The corresponding values in the saline-treated control rats were 164.83 ± 4.23 and 119.1 ± 2.17, respectively (Fig. 1; $n = 6$ in both groups). The stimulus strength required to induce a spike was 7.87V (± 0.98) in LPS-treated rats compared with 4.02V ± 1.2 in saline-injected rats.

The LPS-induced attenuated LTP was associated with a significant increase in reactive oxygen species production, caspase-1 activity, IL-1β concentration, and JNK activity (*$p < 0.05$, ** $p < 0.01$, Student's t test for independent means, Fig. 2, *a–d*; $n = 6$ in all cases) in hippocampus. The stimulatory effect of LPS on JNK activity was mimicked by the addition of IL-1β to hippocampal tissue *in vitro* ($p < 0.01$, Student's t test for paired means, Fig. 2*e*). Fig. 2*f* indicates that endogenous glutamate release was increased significantly by the addition of 40 mM KCl to synaptosomes prepared from untetanized dentate gyrus of saline-pretreated rats (*$p < 0.05$, Student's t test for paired means), but this effect was enhanced in synaptosomes prepared from tetanized dentate gyrus (**$p < 0.01$, Student's t test for paired means). In contrast, KCl failed to stimulate glutamate release in synaptosomes prepared from untetanized dentate gyrus of LPS-pretreated rats, although release was increase in tetanized tissue (*$p < 0.05$, Student's t test for paired means) albeit to an attenuated degree.

Inhibition of Caspase-1 Blocks the Effects of LPS—These data suggested that the LPS-induced effect on LTP may be a consequence of its ability to increase activity of caspase-1 and thence IL-1β concentration and to determine whether this was the case, rats were injected intracerebroventricularly with 5 μl of a caspase-1 inhibitor peptide (Ac-YVAD-CMK) or with 5 μl of saline prior to LPS or saline treatment. Fig. 3*a* indicates that although LTP was inhibited by LPS, this effect was blocked by the caspase-1 inhibitor. Thus the mean percentage change in population epsp slope (± S.E.) in the 2 min immediately after tetanic stimulation was 177.77 ± 15.34 in the control group (treated with saline intracerebroventricularly and intraperitoneally) compared with 118.92 ± 3.35 in the group treated with saline intracerebroventricularly and LPS intraperitoneally. In the last 5 min of the experiment the values were 123.86 ± 2.14 and 96.21 ± 1.14, respectively. Injection of the caspase-1 inhibitor partially reversed the inhibitory effect of LPS on the early changes induced by the tetani and completely blocked the LPS-induced inhibition of the later phase of LTP; the mean percentage changes were 147.32 ± 9.23 and 123.54 ± 6.33 in the first 2 min after tetanic stimulation and in the last 5 min of the experiment, respectively. However, LTP was similar in the control rats and the group of rats injected the caspase-1 inhibitor intracerebroventricularly and saline intraperitoneally; in the latter group, the mean percentage changes in population epsp slope were 174.57 ± 16.24 and 142.07 ± 7.42 in the 2 min after tetanic stimulation and the last 5 min of the experiment, respectively (Fig. 3*a*, $n = 6$ in all groups).

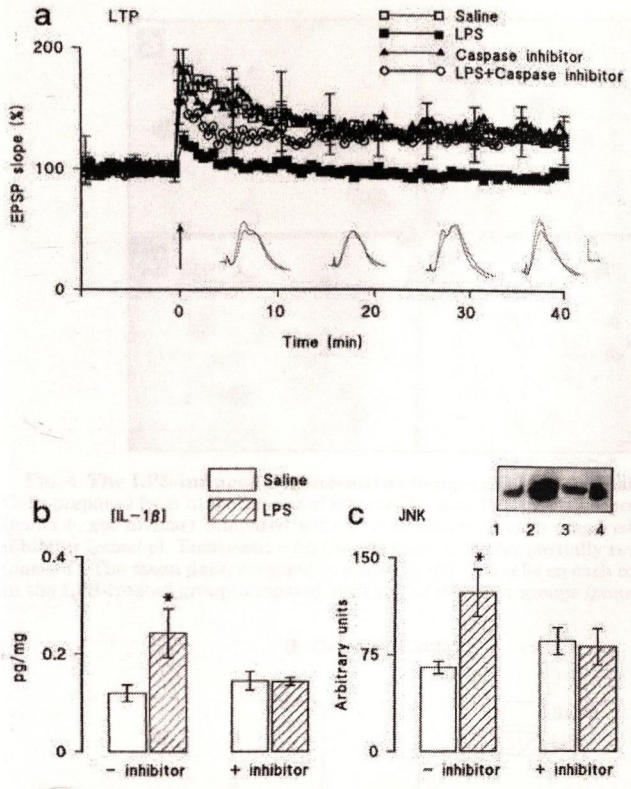

FIG. 3. **The LPS-induced changes in LTP, IL-1β, and JNK activity were inhibited by pretreatment with a caspase-1 inhibitor.** The inhibitory effect of LPS on LTP was blocked by intracerebroventricular injection of the caspase-1 inhibitor, which exerted no significant effect in saline-treated rats (*panel a*). The data presented are the means of six observations in each treatment group; S.E. are included for every 10th response. Sample recordings in the 5 min immediately before tetanic stimulation and in the last 5 min of the experiment are superimposed for saline-injected (*first* and *third traces*) and LPS-injected (*second* and *fourth traces*) rats pretreated with either saline (*first* and *second traces*) or caspase-1 inhibitor (*third* and *fourth traces*). The *scale bars* represent 1 mV and 2 ms. Similar pretreatment with the caspase-1 inhibitor blocked the LPS-induced increases in IL-1β concentration (*panel b*) and JNK activity (*panel c*), whereas treatment with the caspase-1 inhibitor alone exerted no significant effect. A sample immunoblot indicates the stimulatory effect of LPS (*lane 2*) on JNK activity in the absence of the caspase-1 inhibitory (compare *lanes 1* and *2*) and the inhibition of this effect after pretreatment with Ac-YVAD-CMK (compare *lanes 3* and *4*).

Fig. 3*b* shows that intraperitoneal injection of LPS (in rats treated with saline intracerebroventricularly) significantly increased IL-1β concentration in hippocampus (* $p < 0.05$, Student's t test for independent means, $n = 6$) and that this effect was inhibited by pretreatment with the caspase-1 inhibitor. Similarly, JNK activity was enhanced significantly in hippocampal tissue prepared from LPS-treated rats ($p < 0.05$, Student's t test for independent means), but this effect was also inhibited by the caspase-1 inhibitor (Fig. 3*c*).

Inhibition of Caspase-1 Blocks LPS-induced Degenerative Changes in Hippocampus and Entorhinal cortex—Acutely dissociated cells were prepared from hippocampal tissue obtained from rats in each of the four treatment groups. LPS treatment significantly increased the number of degenerating cells, with evidence of an increased number of cells displaying degenerative features such as shrinkage and blebbing of the plasma membrane (Fig. 4*b*). This contrasts with cells prepared from saline-treated rats (*panel a*) and rats treated only with caspase-1 inhibitor (*panel c*). Treatment with the caspase-1 inhibitor (*panel d*) partially reversed the effects of LPS with fewer cells displaying degenerative changes. Fig. 4*e* shows that the percentage of cells which showed degenerative changes was enhanced significantly in the LPS-treated group compared with any of the other groups ($p < 0.05$, Student's t test for independent means) and indicates that the caspase-1 inhibitor reversed the degenerative effect of LPS.

In an effort to account for the compromise in transmitter release observed in dentate gyrus synaptosomes prepared from LPS-treated rats, we analyzed caspase-1 activity and IL-1β concentrations in tissue prepared from entorhinal cortex and found that both measures were increased significantly after LPS treatment (*$p < 0.05$, Student's t test for independent means), but these effects were both attenuated by the caspase-1 inhibitor (Fig. 5, *a* and *b*, $n = 6$). In parallel with the observations in hippocampus, we observed that there was an LPS-induced increase in the number of degenerating cells changes (** $p < 0.01$, Student's t test for independent means, Fig. 6*a*), with evidence of cell shrinkage and membrane blebbing. Pretreatment with the caspase-1 inhibitor blocked these LPS-associated changes (Fig. 6, *a* and *b*). Consistent with the evidence of cell degeneration in hippocampus, we observed that cytochrome *c* translocation was increased markedly in tissue prepared from entorhinal cortex of LPS-treated rats ($p < 0.01$, Student's t test for independent means), whereas there was a decrease in expression of the 116-kDa fragment of (PARP, * $p <$ 0.05, Student's t test for independent means); both of these LPS-associated changes were attenuated by pretreatment with the caspase-1 inhibitor (Fig. 6, *c* and *d*; $n = 6$).

DISCUSSION

We set out to investigate the effect of an intraperitoneal injection of LPS on synaptic function in hippocampus because LPS is considered to contribute significantly to the neuropathological effects associated with Gram-negative bacterial infections probably by increasing IL-1β concentration in brain. The evidence presented indicates that the LPS-induced increase in IL-1β concentration, consequent on increased caspase-1 activity, leads to activation of JNK which may underlie the observed decrease in transmitter release in dentate gyrus, degenerative changes in hippocampus and entorhinal cortex, and inhibition of LTP.

Intraperitoneal injection of LPS inhibited LTP in perforant path granule cell synapses; to our knowledge this effect of LPS has not been shown previously. The current data present at least two possible mechanisms that might underlie the effects. First, we observed that LPS induced an increase in reactive oxygen species production in hippocampus, and the attenuated LTP may arise, directly or indirectly, from this. Such an effect of oxygen radicals has been reported in CA1 *in vitro* (32), and we have recently observed that LTP in dentate gyrus *in vivo* was inhibited by hydrogen peroxide.[2] A second possibility is that the impairment in LTP is a consequence of the LPS-induced increase in IL-1β concentration in hippocampus. We have observed that intracerebroventricular injection of IL-1β inhibits LTP in perforant path-granule cell synapses *in vivo* (14, 15, 33) and that LTP was also compromised in aged and stressed rats, in which hippocampal IL-1β concentration is increased (14). The inhibitory effect of IL-1β on LTP *in vitro* has also been documented; thus IL-1β-induced attenuation of LTP in CA1 (11), CA3 (12) and dentate gyrus (13) has been reported.

Peripheral injection of LPS induced an increase in IL-1β concentration in hippocampus, which supports earlier reports of a similar change in hippocampus and cortex (3, 34), cerebellum (4), and in whole brain (2). These data are backed up by

[2] A. Lynch, P. M., Queenan, and M. A. Lynch, unpublished observation.

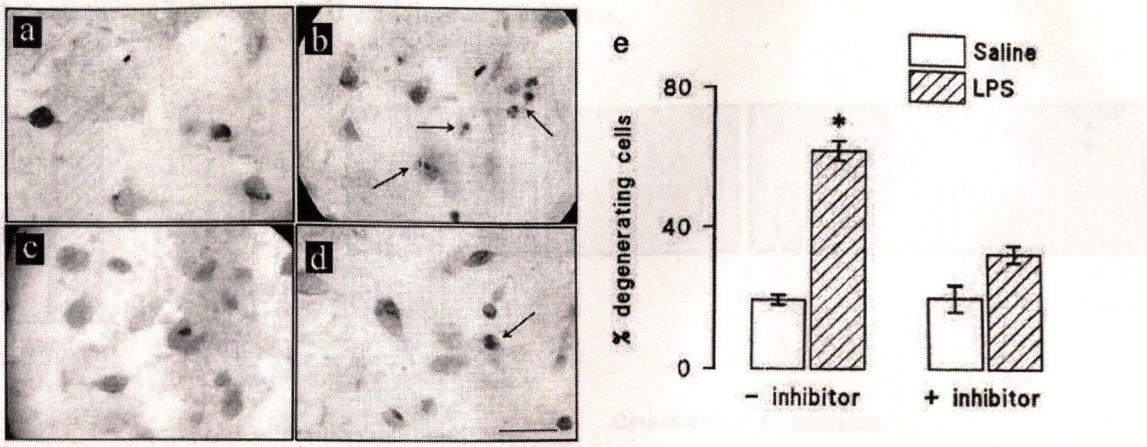

FIG. 4. **The LPS-induced degenerative changes in hippocampal cells were inhibited by pretreatment with the caspase-1 inhibitor.** Cells prepared from hippocampus of rats treated with LPS display degenerative features such as shrinkage and blebbing of the plasma membrane (*panel b*, see *arrows*) compared with no such effects in cells prepared from saline-treated rats (*panel a*) and rats treated only with caspase-1 inhibitor (*panel c*). Treatment with the caspase-1 inhibitor partially reversed the effects of LPS with fewer cells displaying degenerative changes (*panel d*). The mean data, obtained by counting 100–200 cells on each coverslip, show a significant increase in the percentage of degenerating cells in the LPS-treated group compared with any of the other groups (*panel e*, $p < 0.05$, Student's t test for independent means).

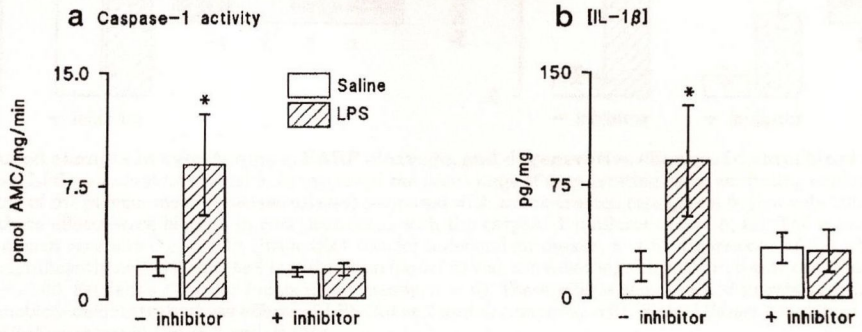

FIG. 5. **The LPS-induced increases in caspase-1 activity and IL-1β concentrations in entorhinal cortex were blocked by the caspase-1 inhibitor.** Caspase-1 activity (*panel a*) and IL-1β concentration (*panel b*) were increased significantly in tissue prepared from entorhinal cortex of LPS-treated rats compared with saline-treated rats (*$p < 0.05$, Student's t test for independent means, $n = 6$). Pretreatment with the caspase-1 inhibitor blocked this effect but alone exerted no significant effect.

several observations of LPS-induced increases in IL-1β concentrations or IL-1β mRNA in cultured glial cells (4, 35), which, together with other data indicating that IL-1β is synthesized in glia (17, 18) and neurons (19, 20), suggests that IL-1β may be produced locally. We have observed that the blood-brain barrier is not affected by intraperitoneal injection of 200 μg/kg LPS within the time frame of the experiment described here,[3] suggesting that the increase in hippocampal IL-1β is unlikely to be peripheral in origin. We cannot rule out the possibility that LPS gains access to the brain after intraperitoneal injection and stimulates production of IL-1β directly, or indirectly, for example through production of tumor necrosis factor or another cytokine. The evidence presented suggests that the increase in IL-1β concentration in hippocampus is a consequence of an increase in caspase-1 activity, which cleaves pro-IL-1β to yield the active cytokine. Cleavage may occur by an autocatalytic process, or it may involve other proteases (21), or, because caspases are cysteine-dependent enzymes and are redox-sensitive (36), they may be stimulated by reactive oxygen species (37). The parallel LPS-induced increases in reactive oxygen species production and caspase-1 activity observed here might indicate a causal interaction between these two parameters. The LPS-induced increase in caspase-1 activity is in contrast to a previous finding in which intraperitoneal injection of 2 mg/kg LPS (compared with 200 μg used here) triggered caspase-1 activation in pituitary gland, but not in hippocampus or hypothalamus (38), although mRNA for caspase-1 was increased in all regions.

JNK phosphorylation was increased significantly in hippocampus of LPS-treated rats, and although a similar effect has been reported in neutrophils (39), macrophages (39, 40), and cultured rat microglia (41), we believe that this is the first indication that such an effect occurs in brain *in vivo*. A few groups have reported that LPS stimulates tyrosine kinase activity, for example in macrophages (42) and in cultures of glial cells (35); this probably represents one of the earliest signaling events stimulated by LPS and explains the observed increase in phosphorylation of mitogen-activated protein kinases, JNK, and p38 (39, 40). The stimulatory effect of LPS on JNK activation *in vivo* was mimicked *in vitro* by IL-1β as reported by us previously (16). Consistent with the evidence presented here, we have observed that LTP in dentate gyrus was attenuated when JNK activity was enhanced in hippocampus, for example in aged rats (16) or rats injected intracerebroventricularly with IL-1β (56).

Several reports indicate that LTP in dentate gyrus is accompanied by an increase in release of glutamate (26, 43–45). The present data provide further direct evidence of the coupling between LTP and enhanced glutamate release and indirect evidence of a causal relationship between these parameters

[3] M. Brady, E. Vereker, and M. A. Lynch, unpublished observation.

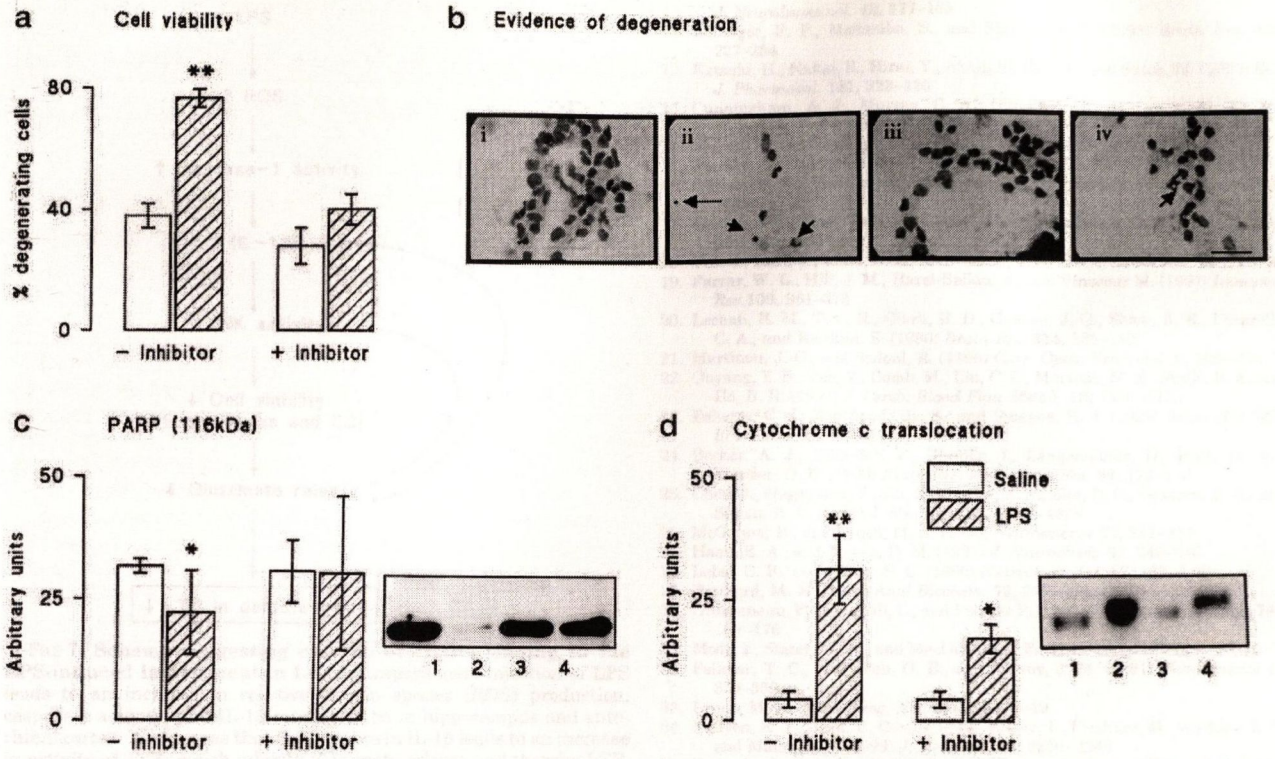

FIG. 6. **The LPS-induced changes in cytochrome *c*, PARP cleavage, and degenerative changes in entorhinal cortex are blocked by the caspase-1 inhibitor.** LPS treatment (see *panel b, ii*) increased the percentage of degenerating cells, exhibiting evidence of degeneration such as shrinkage and blebbing of the plasma membrane (see *arrows*) compared with saline-treated rats (*panel b, i*) or rats treated only with caspase-1 inhibitor (*panel b, iii*); these effects were blocked in rats pretreated with the caspase-1 inhibitor (*panel b, iv*). The mean data were obtained by counting 80–100 cells on each coverslip (*$p < 0.05$, Student's t test for independent means, $n = 6$). Expression of the 116-kDa subunit of PARP (*panel c*) was decreased significantly and cytochrome *c* translocation (*panel d*) was increased significantly in tissue prepared from entorhinal cortex of LPS-treated rats (*$p < 0.05$, Student's t test for independent means, $n = 6$). These effects were blocked in rats pretreated with the caspase-1 inhibitor. Sample immunoblots demonstrate these effects of LPS (*lanes 2* and *4*) compared with control (*lanes 1* and *3*) in saline-pretreated (*lanes 1* and *2*) and Ac-YVAD-CMK-pretreated (*lanes 3* and *4*) rats.

because both were attenuated after LPS treatment. The mechanism underlying the LPS-induced decrease in release was not addressed directly in this study but is paralleled by increased IL-1β concentration and increased JNK activation. An inhibitory effect of IL-1β on glutamate release in hippocampus has been reported previously (46), whereas release has also been shown to be attenuated when IL-1β concentration and JNK activation are increased in hippocampus, for example in aged rats (16).

If increased IL-1β concentration and/or increased JNK activation in hippocampus is primarily responsible for the LPS-induced impairment in LTP, it follows that LTP would not be affected if these changes were inhibited; we argued that this inhibition might be achieved by pretreating rats with the caspase-1 inhibitor Ac-YVAD-CMK. We report that the inhibitor blocked each of these effects of LPS, providing evidence that they are causally linked and permitting us to restate our proposal that LTP is impaired when IL-1β concentration is increased (14, 15) and adding support to the argument that increased JNK activation is coupled with impaired LTP (16).

There was an increase in the number of cells exhibiting evidence of degeneration in both entorhinal cortex and hippocampus prepared from LPS-treated rats compared with saline-treated controls. Because this was reversed by the caspase-1 inhibitor, it seems likely that one of the consequences of increased activity of caspase-1, for example increased concentration of IL-1β or increased activation of JNK, mediates the effect. A role for the caspases in cell death has been described (21), and increased concentrations of IL-1β have been closely linked with neuronal degeneration (6–10, 47). Similarly, JNK activation has been shown to be a component of the cell death pathway in PC12 cells (48–50) and is required for apoptosis during early brain development (51).

Accompanying the LPS-associated neuronal degeneration in entorhinal cortex, we observed an increase in caspase-1 activity and IL-1β concentration, both of which were attenuated in tissue prepared from rats pretreated with the caspase-1 inhibitor. Evidence of increased cytochrome *c* translocation and cleavage of the DNA repair enzyme, PARP, were also observed after LPS treatment. Cytochrome *c* translocation from the mitochondria to the cytosol, which may trigger activity of certain caspases (52), has been shown to be associated with apoptosis (53). This was confirmed by the recent demonstration that injection of cytochrome *c* (but not an inactive cytochrome *c*) resulted in apoptotic morphology in a variety of cells (54) and also by the finding that hydrogen peroxide induces apoptosis by triggering cytochrome *c* release from mitochondria (55). Similarly, cleavage of PARP, a substrate for caspases, has also been considered to be a reliable indicator of apoptosis (21). It is significant, though not perhaps surprising, that the LPS-induced increases in cytochrome *c* translocation and PARP cleavage were inhibited in tissue prepared from rats pretreated with the caspase-1 inhibitor. These data therefore suggest a role for caspase-1 in neuronal degeneration.

We attribute the decrease in LTP after injection of LPS described here to degenerative changes in neuronal cells in both the hippocampus and entorhinal cortex. Because these changes are blocked by a caspase-1 inhibitor, it seems that

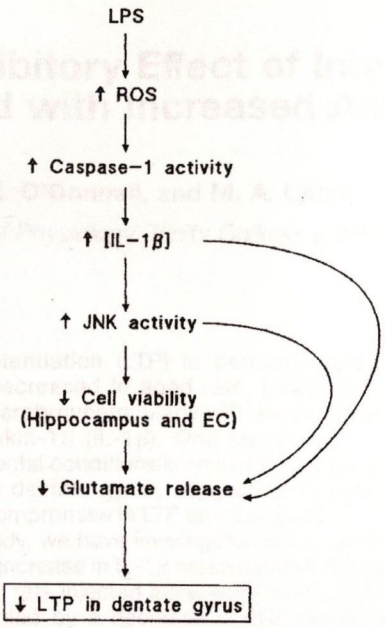

FIG. 7. **Scheme suggesting cascade of events leading to the LPS-induced impairment in LTP.** Intraperitoneal injection of LPS leads to an increase in reactive oxygen species (*ROS*) production, caspase-1 activity, and IL-1β concentration in hippocampus and entorhinal cortex. We propose that the increase in IL-1β leads to an increase in activity of JNK, which inhibits glutamate release and thereby LTP. The evidence presented suggests that increased IL-1β concentration also leads to degenerative changes in both hippocampus and entorhinal cortex, which is likely to contribute to the impairment in LTP.

IL-1β plays a pivotal role. The evidence suggests that LPS injection increases caspase-1 activity and thence IL-1β expression in hippocampus and entorhinal cortex. We propose that one downstream effect of increased IL-1β concentration in hippocampus is an increase in JNK phosphorylation, which directly or indirectly inhibits glutamate release (Fig. 7); however, the data presented are consistent with the view that increased IL-1β concentration in hippocampus and entorhinal cortex leads to degenerative changes consistent with apoptosis, and these changes are likely to contribute to the impaired LTP exhibited by LPS-treated rats.

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The Inhibitory Effect of Interleukin-1β on Long-Term Potentiation Is Coupled with Increased Activity of Stress-Activated Protein Kinases

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Long-term potentiation (LTP) in perforant path-granule cell synapses is decreased in aged rats, stressed rats, and rats injected intracerebroventricularly with the proinflammatory cytokine interleukin-1β (IL-1β). One factor that is common to these experimental conditions is an increase in the concentration of IL-1β in the dentate gyrus, suggesting a causal relationship between the compromise in LTP and increased IL-1β concentration. In this study, we have investigated the downstream consequences of an increase in IL-1β concentration and report that the reduced LTP in rats injected intracerebroventricularly with IL-1β was accompanied by a decrease in KCl-stimulated glutamate release in synaptosomes prepared from dentate gyrus, although unstimulated glutamate release was increased. These changes were paralleled by increased activity of the stress-activated kinases, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase. Intracerebroventricular injection of IL-1β increased reactive oxygen species production in hippocampal tissue, whereas IL-1β and H_2O_2 increased activities of both JNK and p38 *in vitro*. Dietary manipulation with antioxidant vitamins E and C blocked the increase in reactive oxygen species production, the stimulation of JNK and p38 activity, the attenuation of glutamate release, and the IL-1β-induced inhibitory of LTP. We propose that IL-1β stimulates activity of stress-activated kinases, which in turn may inhibit glutamate release and result in compromised LTP and that these actions are a consequence of increased production of reactive oxygen species.

Key words: LTP; dentate gyrus; IL-1β; stress-activated kinases; glutamate release; reactive oxygen species

Consistent with the high expression of IL-1 receptors in the hippocampus (Lechan et al., 1990; Ban et al., 1991; Parnet et al., 1994) are several observed effects of exogenous IL-1 in this brain area. For example, IL-1β exerts an inhibitory effect on (1) long-term potentiation (LTP) in CA1 (Bellinger et al., 1993), CA3 (Katsuki et al., 1990), and dentate gyrus (Cunningham et al., 1996; Murray and Lynch, 1998a,b), (2) release of acetylcholine (Rada et al., 1991) and glutamate (Murray et al., 1997) in hippocampal synaptosomes, (3) calcium influx in hippocampal synaptosomes (Murray et al., 1997), and (4) Ca^{2+} channel currents in hippocampal neurons (Plata-Salaman and ffrench-Mullen, 1994).

The mechanism by which IL-1β inhibits LTP remains to be established. Because maintenance of LTP has been associated with increased glutamate release (Bliss and Collingridge, 1993; Canevari et al., 1994; McGahon and Lynch, 1996; McGahon et al., 1997), one factor that may contribute to inhibition of LTP is the inhibitory effect of IL-1β on glutamate release. However, it has been recently reported that the IL-1β-induced attenuation of LTP in dentate gyrus *in vitro* is blocked by SB203580 (Coogan et al., 1997), an inhibitor of p38 that is one member of the family of mitogen-activated protein (MAP) kinases. The MAP kinase family has been identified as a major player in cellular signaling and markedly influences such diverse processes as cell proliferation, cell differentiation, and cell death. Consistent with the evidence that nerve growth factor and other growth factors stimulate extracellular signal-regulated protein kinase (ERK), is the generally held view that activation of this particular pathway results in neurite outgrowth, cell proliferation, or differentiation (Seger and Krebs, 1995; Xia et al., 1995; Creedon et al., 1996). In contrast, c-Jun N-terminal kinase (JNK) and p38 are activated by environmental stress, including oxidative stress (Raingeaud et al., 1995; Uciechowski et al., 1996; Junger et al., 1997), and activation leads to growth arrest or even cell death (Park et al., 1996; Maroney et al., 1998). JNK and p38 are stimulated by IL-1β (Derijard et al., 1994; Raingeaud et al., 1995; Rizzo and Carlo-Stella, 1996; Uciechowski et al., 1996; Lu et al., 1997), which is consistent with the observation that IL-1β has been implicated in cell death (Rothwell, 1999). In general, the evidence that indicates that IL-1β activates JNK and p38 and that activation of these pathways induces cell damage or cell death has been obtained in various circulating and cultured cells, whereas evidence for similar changes in neuronal tissue is lacking.

In this study we have investigated the downstream consequences of an increase in IL-1β in hippocampal tissue and report that this cytokine increases activity of both JNK and p38. We also provide evidence that IL-1β-induced activation of JNK and p38 leads to a decrease in glutamate release and might be responsible for the attenuation in both the early and later components of LTP observed in rats which received an intracerebroventricular injection of IL-1β.

MATERIALS AND METHODS

Animals. Groups of male Wistar rats (300–350 gm) were used in these experiments. Animals were housed in groups of two to four under a 12 hr light/dark schedule. Ambient temperature was controlled between 22 and 23°C. Food and water were available *ad libitum*. In some experiments food and water intake was measured daily for 1 week, and at the end of this period rats were randomly subdivided into two groups. One group received normal laboratory chow with added vitamin E (250 mg of DL-α-tocopheryl acetate per rat per day, dissolved in corn oil; Beeline Healthcare, Dublin, Ireland). The laboratory chow contained 3.5% crude oil and 55 mg/kg vitamin E; thus average daily intake of vitamin E from this diet was 2.75 mg/rat. Ascorbic acid (250 mg/rat per day) was added to the water given to these rats. The second group received normal laboratory chow with corn oil added to ensure isocaloric intake with the first group. Rats were offered 100% of their average daily food and average water intakes so that the full daily allowances of vitamins would be ingested. Diet was prepared freshly each day. Food and water intake did not vary between groups, and there was no significant difference in daily food and water intake before and after dietary modifications were made. Rats were fed on the respective control or supplemented diet for 5 d and were under veterinary supervision for the duration of this experiment.

Induction of LTP in perforant path-granule cell synapses in vivo. LTP was induced as described previously (McGahon and Lynch, 1996). Rats were anesthetized by intraperitoneal injection of urethane (1.5 gm/kg), placed in a head holder in a stereotaxic frame, and injected intracerebroventricularly with either IL-1β (5 μl; 3.5 ng/ml; human recombinant; 5×10^{-7} U/mg; The Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD) or saline (5 μl). [We measured deep body

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temperature immediately before injection and at 15 min intervals after injection for 90 min. The temperature increased slightly from 35.18°C (±1.29, SEM, $n = 5$) to 36.05°C (± 2.08) in saline-injected rats and 35.08°C (±0.32) to 35.22°C (±0.61) in IL-1β-treated rats during the course of the treatments.] A window of skull was removed to allow placement of recording and stimulating electrodes in the molecular layer of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma) and perforant path, respectively (angular bundle, 4.4 mm lateral to lambda). The depth of the electrodes was adjusted to obtain maximal responses in the cell body region. Stable baseline recordings were recorded for ~15 min, and electrophysiological recording commenced 30 min after intracerebroventricular injection; this was ~60 min after administration of urethane. Test shocks at the rate of 1/30 sec were delivered for 10 min before and 40 min after tetanic stimulation (three trains of stimuli; 250 Hz for 200 msec; intertrain interval 30 sec). In a separate series of experiments, a group of four rats received tetanic stimulation, and another group of four received the same total number of stimuli, but no high-frequency train of stimuli, i.e., one stimulus every 12 sec. At the end of the electrophysiological recording period, rats were killed by decapitation, the hippocampus was removed, and the tetanized and untetanized dentate gyri, as well as the hippocampus proper, were dissected on ice and cross-chopped into slices (350 × 350 μm) using a McIlwain tissue chopper. The time taken to prepare slices from the time of death was 2.5–3.5 min. All samples were frozen separately in 1 ml of Krebs' solution (composition of Krebs' in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, and $CaCl_2$ 1.13) containing 10% dimethylsulfoxide according to the method of Haan and Bowen (1981). For analysis, thawed slices of untetanized and tetanized dentate gyrus were rinsed three times in fresh ice-cold Krebs' solution and homogenized in 1 ml of ice-cold sucrose (0.32 M) for preparation of P_2 (McGahon and Lynch, 1996), which was used for analysis of glutamate release and activity of p38 and JNK. Slices of hippocampus were rinsed and homogenized in either 200 μl of fresh Krebs' solution for analysis of vitamins E and C and superoxide dismutase activity or 40 mM Tris-HCl, pH 7.4, for analysis of reactive oxygen species production.

Release of glutamate. The impure synaptosomal preparation P_2 was resuspended in oxygenated Krebs' solution containing 2 mM $CaCl_2$ (McGahon and Lynch, 1996), and glutamate release was assessed as described previously (McGahon et al., 1999). Briefly, synaptosomal tissue was aliquoted onto Millipore (Bedford, MA) filters (0.45 μm), rinsed under vacuum, and the filtrate was discarded. Synaptosomes were then incubated in 250 μl of oxygenated Krebs' solution at 37°C for 3 min, in the presence or absence of 40 mM KCl, and filtrate was collected and stored for analysis as described (Ordronneau et al., 1991). Triplicate samples (50 μl) or glutamate standards (50 μl; 25 nM to 1 μM prepared in 100 mM Na_2HPO_4 buffer, pH 8.0) were added to glutaraldehyde-coated 96-well plates, incubated for 60 min at 37°C, and washed with 100 mM NaH_2PO_4 buffer. Ethanolamine (250 μl; 0.1 M in 100 mM Na_2HPO_4 buffer) was used to bind unreacted aldehydes and donkey serum (200 μl; 3% in PBS-T) was added to block nonspecific binding. Samples were incubated overnight at 4°C in the presence of antiglutamate antibody (raised in rabbit; 100 μl; 1:5000 in PBS-T; Sigma, Poole, UK), washed and reacted with secondary antibody [anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody; 100 μl; 1:10,000 in PBS-T; Amersham, UK] for 60 min at room temperature. 3,3′,5,5′-Tetramethylbenzidine liquid substrate was added as chromogen, and incubation continued for exactly 60 min at room temperature, at which time the reaction was stopped by H_2SO_4 (4 M; 30 μl). Optical densities were determined at 450 nm using a multiwell plate reader, and values were calculated with reference to the standard curve, corrected for protein (Bradford, 1976) and expressed as micromoles of glutamate per milligram of protein.

Analysis of the activity of the MAP kinases. The activities of JNK and p38 were analyzed in P_2 preparations obtained from frozen hippocampal slices obtained from saline-pretreated and IL-1β-pretreated rats, some of which were fed on control and experimental diets. Activity of the kinases was also assessed by preincubating samples for 20 min in the absence and presence of IL-1β (10 pg/ml) and in the absence and presence of H_2O_2 (5 mM); these experiments were performed in P_2 obtained from freshly prepared hippocampus. In all experiments, samples were analyzed for protein, diluted to equalize for protein concentration, and these samples of synaptosomal protein (10 μl, 1 mg/ml) were added to 10 μl of sample buffer (Tris-HCl, 0.5 mM, pH 6.8; glycerol 10%; SDS, 10%; β-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v), boiled for 5 min, and loaded onto gels (10% SDS for p38 and 12% for JNK). Proteins were separated by application of 30 mA of constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min), and immunoblotted with the appropriate antibody. Proteins were immunoblotted with antibodies that specifically target phosphorylated JNK [Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 in PBS–Tween (0.1% Tween 20) containing 2% nonfat dried milk] or phosphorylated p38 [Santa Cruz Biotechnology; 1:500 in PBS–Tween (0.1% Tween 20) containing 2% nonfat dried milk] by incubating for 2 hr at room temperature. Nitrocellulose strips were washed and incubated for 2 hr at room temperature with secondary antibody [peroxidase-linked anti-mouse IgG; 1:1000 dilution (Sigma) in the case of JNK and peroxidase-linked anti-mouse IgM; 1:1,000 dilution (Amersham) in the case of p38]. Visualization was achieved by ECL detection (Amersham); immunoblots were exposed to film overnight and processed using a Fuji x-ray processor. Quantification of protein bands was achieved by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber, version 2.04.7, Synotics; UVP Ltd) and Gelworks (Gelworks ID, version 2.51; UVP Ltd) for photography and densitometry, respectively. Gelworks provides a single value (in arbitrary units) representing the density of each blot, and the values presented here are means of data generated from at least four separate experiments. The antibodies used in these experiments were specific as judged by the fact that only one band was observed after ECL detection.

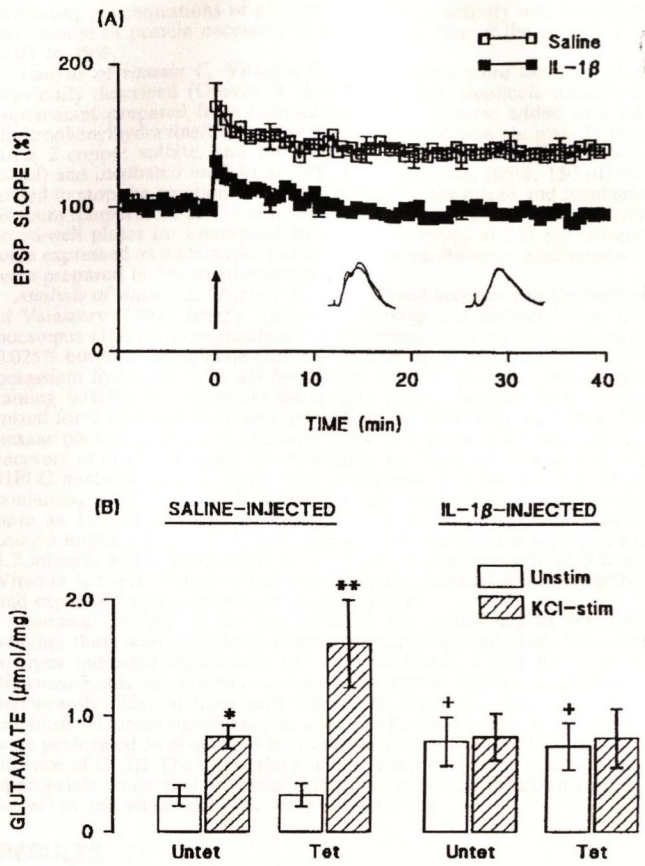

Figure 1. Intracerebroventricular injection of IL-1β inhibited LTP and the associated increase in glutamate release. *A*, Tetanic stimulation induced an immediate increase in EPSP slope in both saline-injected and IL-1β-injected rats, although this was attenuated after IL-1β injection. Mean EPSP slope decreased in IL-1β-treated rats so that the value was close to baseline at the end of the 40 min recording period. Sample recordings in the 5 min immediately before tetanic stimulation and in the last 5 min of the experiment are superimposed for saline-injected (*left-hand* records) and IL-1β-injected (*right-hand* record) rats. SEM values are included for every 10th response. *B*, Endogenous glutamate release was significantly increased in synaptosomes prepared from untetanized dentate gyrus (*Untet*) of saline-injected rats by addition of 40 mM KCl to incubation medium (*$p < 0.05$; ANOVA), but this was enhanced to a greater degree in synaptosomes prepared from tetanized dentate gyrus (*Tet*; **$p < 0.01$; ANOVA). Injection of IL-1β increased unstimulated release in synaptosomes prepared from both untetanized and tetanized dentate gyrus (+$p < 0.05$; ANOVA; compared with the values obtained from saline-injected rats), but addition of 40 mM KCl to the incubation failed to enhance glutamate release in these preparations. The data are means (± SEM) of six individual experiments.

Analysis of reactive oxygen species formation. Formation of reactive oxygen species was assessed by the method of Lebel and Bondy (1990), which relies on the measurement of 2′7′-dichlorofluorescein (DCF), the oxidized, fluorescent product of 2′7′-dichlorofluorescin diacetate (DCFH-DA). Assessments were made in synaptosomes prepared from hippocampus of saline-injected and IL-1β-injected rats and also in synaptosomes prepared from freshly dissected hippocampus (in which the effects of incubating in the presence/absence of IL-1β and glutamate were assessed). Synaptosomes were incubated at 37°C for 15 min in the presence of DCFH-DA (10 μl; 5 μM, from a stock of 500 μM in methanol). To terminate the reaction, the dye-loaded suspensions were centrifuged at 13,000 × *g* for 8 min at 4°C, and the pellets were resuspended in 2 ml of ice-cold 40 mM Tris buffer, pH 7.4, and monitored for fluorescence at 37°C with the excitation wavelength at 488 nm and the emission wavelength at

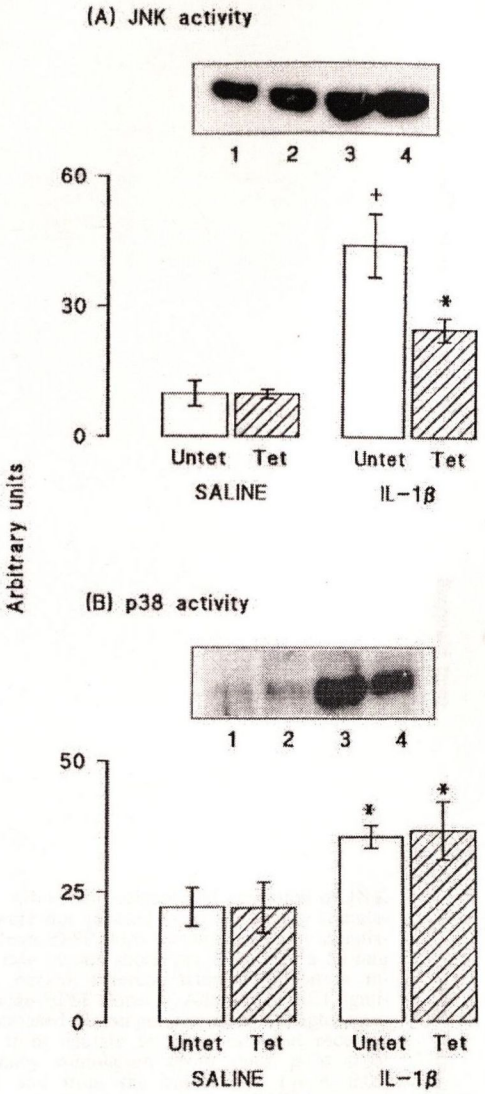

Figure 2. IL-1β increased activities of JNK and p38 in synaptosomes prepared from dentate gyrus. *A,* JNK activity was significantly increased in synaptosomes prepared from untetanized and tetanized (*$p < 0.05$; +$p < 0.01$; ANOVA) dentate gyrus of IL-1β-injected rats compared with either untetanized or tetanized tissue prepared from saline-injected rats. JNK activity was reduced in tetanized, compared with untetanized, tissue after IL-1β injection but was similar in the two preparations obtained from saline-injected rats. *B,* Activity of p38 tissue was significantly increased in synaptosomes prepared from untetanized and tetanized dentate gyrus of IL-1β-injected rats compared with either untetanized and tetanized tissue obtained from saline-injected rats (*$p < 0.05$; ANOVA). Tetanic stimulation did not affect enzyme activity in tissue prepared from saline- or IL-1β-treated rats. The data are means of six (± SEM) individual experiments. Sample immunoblots in *A* and *B* demonstrate kinase activities in untetanized and tetanized tissue prepared from saline-treated (*lanes 1* and *2*) and IL-1β-treated (*lanes 3* and *4*) rats.

525 nm. In some experiments IL-1β (1 ng/ml) or glutamate (50 μM or 250 μM) was included in the incubation to medium to assess its effect of reactive oxygen species production. Results were expressed as micromoles of DCF formed per milligram of protein from a DCF standard curve (0.05–1 μM).

Analysis of superoxide dismutase activity. Superoxide dismutase activity was determined according to the method described by Spitz and Oberley (1989). Aliquots (800 μl) of incubation buffer [50 mM potassium buffer, pH 7.8, containing 1.8 mM xanthine, 2.24 mM nitroblue tetrazolium (NBT), 40 U of catalase, 7 μl/ml xanthine oxidase, and 1.33 mM diethylenetriaminepentacetic acid] were added to 1.5 ml microfuge tubes containing samples of supernatant prepared from hippocampal tissue (100 μl) at different dilutions (1:2, 1:5, 1:10, 1:20, 1:50, and 1:100) and analyzed by UV spectroscopy at 560 nm. Slices were homogenized, and enzyme activity was assessed as the rate of reduction of NBT, which was inhibited with increasing concentrations of protein. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%.

Analysis of vitamin C. Vitamin C concentrations were determined as previously described (Omaye et al., 1979). Briefly, duplicate aliquots of supernatant prepared from hippocampus (100 μl) were added to a 2,4-dinitrophenylhydrazine/thiourea/copper (DTC) solution (in mM: 50 thiourea, 2 copper sulfate, and 150 dinitrophenylhydrazine in 9 N H_2SO_4; 20 μl) and incubated for 3 hr at 37°C. Ice-cold H_2SO_4 (65%; 150 μl) was added to stop the reaction, and samples were vortex-mixed and incubated at room temperature for 30 min before aliquots (100 μl) were transferred to 96-well plates for assessment by UV spectroscopy at 545 nm. Results were expressed as micromoles per gram of tissue. Ascorbic acid standards were prepared in 5% trichloroacetic acid.

Analysis of vitamin E. Vitamin E was analyzed according to the method of Vatassery (1994). Briefly, aliquots of homogenate prepared from hippocampus (150 μl) were incubated in the presence of ethanol containing 0.025% butylhydroxytoluene (150 μl), 25% ascorbic acid (70 μl), and 10% potassium hydroxide (135 μl) for 30 min at 60°C. Hexane (540 μl) containing 0.025% butylhydroxytoluene was added, samples were vortex-mixed for 1 min, and they were centrifuged at 1500 rpm for 6 min. The hexane phase was removed and evaporated to dryness under nitrogen; the recovery of vitamin E using this procedure was between 70 and 80%. For HPLC analysis, dried samples were resuspended in methanol (150 μl) containing 0.025% butylhydroxytoluene, and 30 μl volumes were injected onto an Intersil C18 column. Separation of α-tocopherol was achieved using a mobile phase of 75% acetonitrile: 25% methanol at a flow rate of 1.2 ml/min, and samples were detected by UV spectroscopy at 292 nm. Vitamin E concentration was estimated by the external standard method and expressed as nanomoles per gram of tissue.

Statistical analysis. A one-way ANOVA was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), a *post hoc* Student-Newman-Keuls test analysis was used to determine which conditions were significantly different from each other. The Student's *t* test was used to establish statistical significance in some cases; for example, when analyses were performed on aliquots of the same tissue incubated in the presence or absence of IL-1β. The use of the Student's *t* test for paired values was also appropriate when analyses were performed on tissue prepared from untetanized and tetanized tissue from the same rat.

RESULTS

Intracerebroventricular injection of IL-1β inhibited LTP in perforant path-granule cell synapses. The mean percentage increases in EPSP slope in the 2 min immediately after tetanic stimulation (compared with the mean value in the 5 min immediately before tetanic stimulation) were 158.6 ± 6.2 and 120.3 ± 3.1 in the saline-pretreated and IL-1β-pretreated groups, respectively ($n = 6$ in each case). The mean percentage increases in EPSP slope in the last 5 min of the experiment were 139.3 ± 1.6 and 106.2 ± 1.3 in the saline-pretreated and IL-1β-pretreated groups, respectively (Fig. 1*A*).

Analysis of endogenous glutamate release in synaptosomes prepared from tetanized and untetanized tissue obtained from these rats (six rats per group) revealed significant effects of IL-1β injection. Figure 1*B* shows that addition of 40 mM KCl to synaptosomes prepared from untetanized dentate gyrus obtained from saline-treated rats, significantly increased glutamate release ($p < 0.05$; ANOVA), but a further enhancement of release was observed in synaptosomes prepared from tetanized tissue ($p < 0.01$; ANOVA). In contrast, KCl failed to stimulate glutamate release in synaptosomes prepared from both untetanized and tetanized tissue obtained from IL-1β-pretreated rats (Fig. 1*B*). In this experiment, we observed that unstimulated glutamate release was significantly greater in tissue prepared from IL-1β-pretreated rats compared with saline-treated animals ($p < 0.05$; ANOVA). However, although an increase in unstimulated glutamate release was also observed in a separate experiment (see Fig. 7*A*), the increase did not reach statistical significance on this second occasion.

Activities of JNK and p38 were assessed in aliquots of synaptosomal tissue prepared from the hippocampus of the same group of rats. Figure 2 demonstrates that tetanic stimulation did not affect JNK (*A*) or p38 (*B*) activity in tissue prepared from saline-pretreated rats. Both the sample immunoblot and the mean data obtained from densitometric analysis indicate that the activities of both kinases were enhanced in tissue prepared from IL-1β-pretreated rats (*$p < 0.05$; †$p < 0.01$; ANOVA). In the case of p38 activity, the increase was similar in untetanized and tetanized

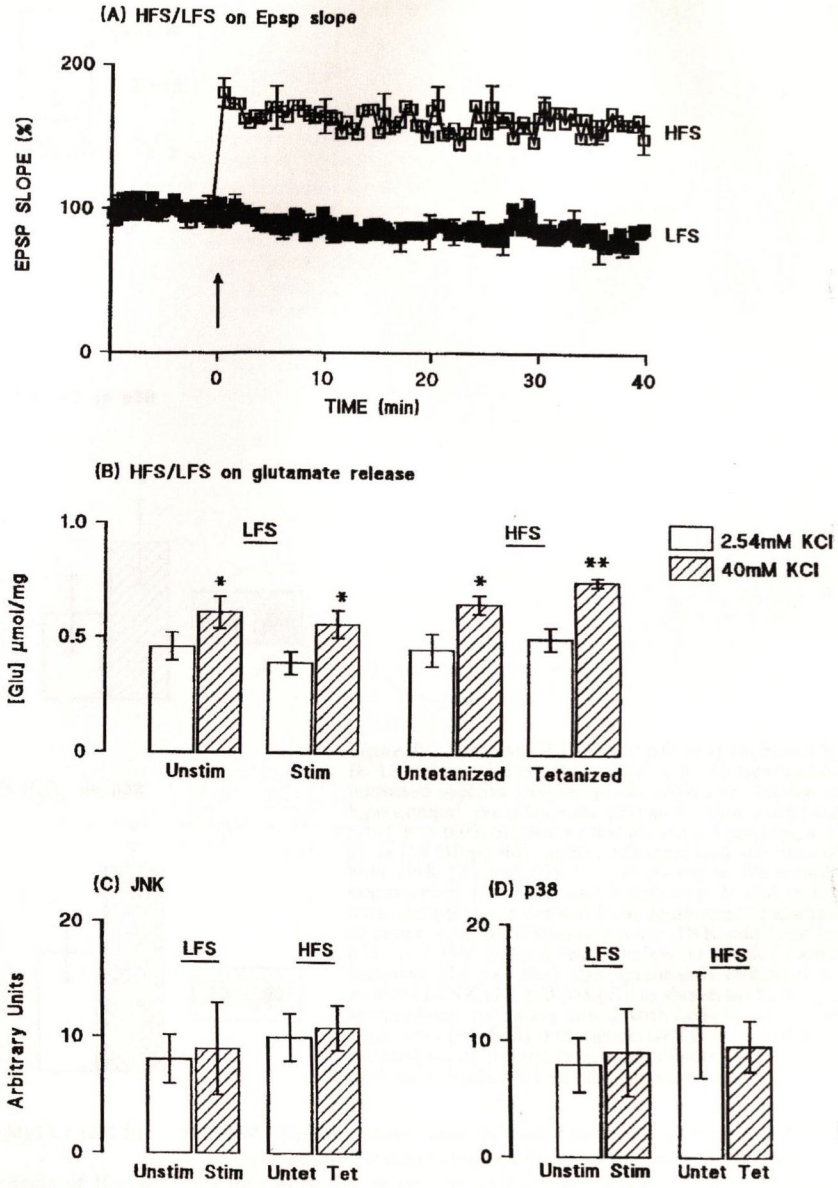

Figure 3. Glutamate release and activation of JNK and p38 were not affected by low-frequency stimulation. *A,* Mean EPSP slope was unchanged by stimulation at a rate of one shock per 12 sec for a 50 min recording period, whereas tetanic stimulation increased mean EPSP slope. *B*, Addition of KCl significantly increased glutamate release in synaptosomes prepared from dentate gyrus of rats that received low-frequency stimulation (both sides; $p < 0.05$; ANOVA) and from the untetanized ($p < 0.05$; ANOVA) and tetanized ($p < 0.01$; ANOVA) tissue of rats that received tetanic stimulation. *C, D,* Activation of JNK and p38 were similar in all preparations. Values are means (± SEM) of four separate experiments for JNK and three for p38.

tissue, but in the case of JNK, the increase was more marked in untetanized, compared with tetanized, tissue (*$p < 0.05$; ANOVA). To confirm that the changes observed in glutamate release and activities of JNK and p38 were, in fact, LTP-associated, we compared these measures in ipsilateral and contralateral dentate gyri prepared from rats that received tetanic stimulation (as described above) or that received the same total number of stimuli to the perforant path but without the high-frequency train of stimuli. Figure 3*A* shows that stimulation at a rate of one shock per 12 sec (low-frequency stimulation) for 50 min did not significantly affect EPSP slope. Figure 3*B* indicates that addition of KCl stimulated glutamate release to a similar extent in synaptosomes prepared from dentate gyrus of rats that received low-frequency stimulation (both sides) and from the untetanized tissue of rats that received tetanic stimulation ($p < 0.05$ in all cases; $n = 4$; ANOVA); however, release was enhanced in synaptosomes prepared from tetanized dentate gyrus ($p < 0.01$; ANOVA). Figure 3, *C* and *D,* indicates that activation of JNK and p38 were similar in all preparations.

Previous studies have suggested that IL-1β might induce an increase in reactive oxygen species production, and because activities of JNK and p38 have been shown to be increased by both reactive oxygen species and IL-1β in some cell types, we investigated these changes in hippocampal tissue. Figure 4*A* shows that incubation of hippocampal synaptosomes in the presence of IL-1β (1 ng/ml) *in vitro* significantly increased reactive oxygen species production ($p < 0.05$; Student's *t* test for paired values). Because IL-1β may increase unstimulated glutamate release, we assessed the effect of glutamate (50 and 250 μM) on reactive oxygen species production *in vitro* and found that it was without effect (data not shown). Figure 4*B–E* shows sample immunoblots and the mean data derived from densitometric analysis in seven separate experiments in which the effects of IL-1β (10 pg/ml) and H_2O_2 (5 mM) were assessed on activity of JNK and four separate experiments in which effects on p38 activity were assessed. Analysis of the mean data indicated that IL-1β significantly increased activities of both JNK and p38 ($p < 0.05$ in each case; Student's *t* test for paired values; Fig. 4*B,C*). Although in the case of p38, SEM values in control and IL-1β-treated tissue overlapped, the difference between the mean values was significant because IL-1β increased p38 phosphorylation in all experiments. H_2O_2, like IL-1β, significantly

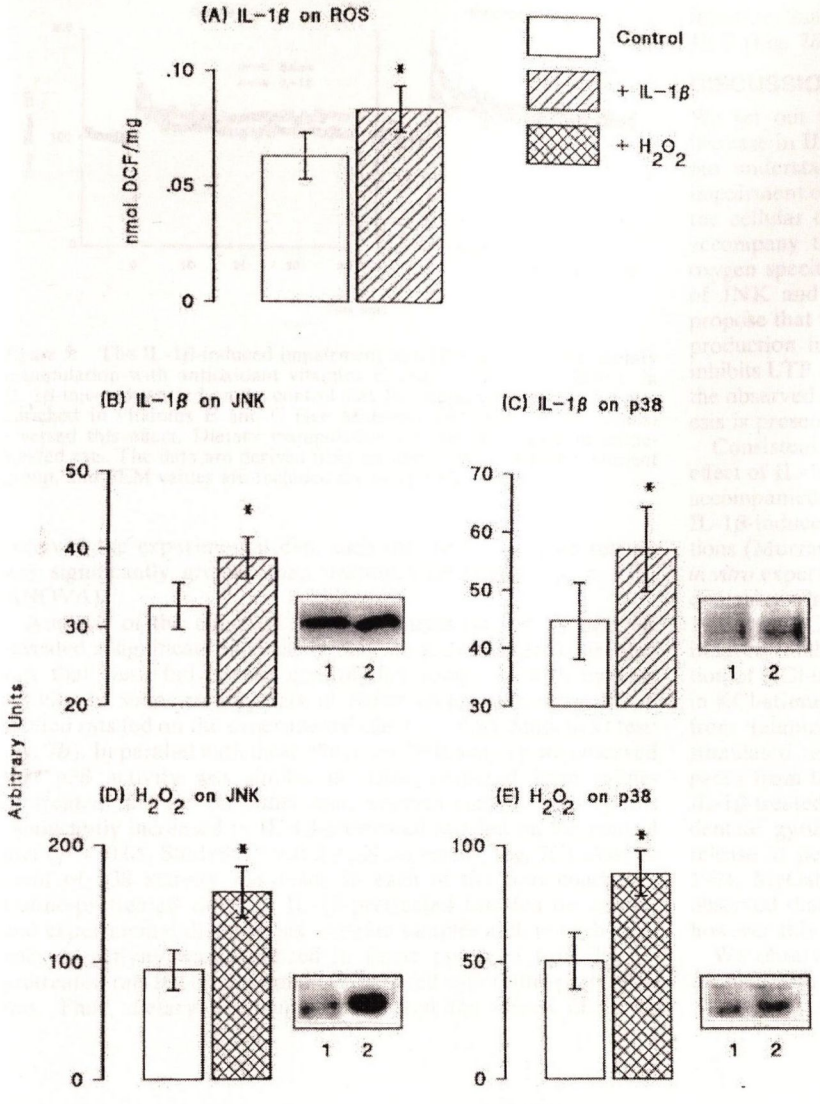

Figure 4. Activities of JNK and p38 were increased by IL-1β and reactive oxygen species. *A*, IL-1β significantly increased reactive oxygen species (*ROS*) production in hippocampal synaptosomes prepared from untreated rats (*$p < 0.05$; Student's *t* test for paired samples; $n = 6$). IL-1β (10 pg/ml) significantly increased activities of both JNK (*B*) and p38 (*C*), as shown in the sample immunoblots (compare *lane 2* with *lane 1*) and in the mean data that was derived from densitometric analysis of seven separate immunoblots for JNK and four for p38 ($p < 0.05$ in each case; Student's *t* test for paired samples). H_2O_2 (5 mM) also significantly increased activities of JNK (*D*) and p38 (*E*), as shown in the sample immunoblots (compare *lane 2* with *lane 1*) and in the mean data (± SEM) that was derived from densitometric analysis of six separate immunoblots ($p < 0.05$ in each case; Student's *t* test for paired samples).

increased activities of both kinases ($p < 0.05$; Student's *t* test for paired values; Fig. 4*D,E*).

These data suggested that at least some of the effects of IL-1β might arise from its stimulatory effect on reactive oxygen species production; if this is the case, it might be predicted that the effect of IL-1β will be inhibited by antioxidants. We therefore assessed the effect of intracerebroventricular injection of IL-1β on LTP in rats fed on control diet and on a diet enriched in antioxidant vitamins E and C. Figure 5 demonstrates that the IL-1β-induced impairment in LTP was absent in rats which were fed on the vitamin-enriched diet. In these experiments, the mean percentage increases in EPSP slope in the 2 min immediately after tetanic stimulation (compared with the mean value in the 5 min immediately before to tetanic stimulation) were 143.2 ± 5.6 and 127.1 ± 2.0 in the saline-pretreated and IL-1β-pretreated groups fed on the control diet and 141.0 ± 3.7 and 172.7 ± 9.7 in the saline-pretreated and IL-1β-pretreated groups fed on the vitamin-enriched diet. The corresponding mean percentage increases in EPSP slope in the last 5 min of the experiment were 120.1 ± 1.2 and 107.5 ± 1.6 and 119.3 ± 3.5 and 125.8 ± 2.3 in the saline- and IL-1β-pretreated groups fed on control and vitamin-enriched diets, respectively (Fig. 5); the difference between the values in the two groups fed on the vitamin-enriched diet did not reach statistical significance.

To assess the changes that accompanied dietary manipulation, we analyzed the activity of superoxide dismutase, the formation of reactive oxygen species, and the concentrations of vitamins E and C in hippocampal tissue prepared from saline- and IL-1β-injected rats that were fed on control and vitamin-enriched diets. Figure 6 shows that IL-1β significantly increased activity of superoxide dismutase in tissue prepared from rats fed on both control and experimental diets ($p < 0.05$; Student's *t* test for paired samples; Fig. 6*A*). We observed that intracerebroventricular injection of IL-1β resulted in a significant increase in reactive oxygen species production in hippocampal synaptosomes prepared from rats fed on the control diet ($p < 0.05$; Student's *t* test for independent means) but that dietary manipulation reversed this effect (Fig. 6*B*). There was a significant increase in vitamin C concentration in hippocampus of rats fed on the experimental, compared with the control, diet ($p < 0.05$; Student's *t* test for independent means; Fig. 6*C*), but vitamin E concentration was unaffected by dietary manipulation (Fig. 6*D*); IL-1β pretreatment did not alter the concentration of either vitamin.

Glutamate release and the activities of JNK and p38 were assessed in synaptosomes prepared from dentate gyrus of saline- and IL-1β-treated rats that were fed on the control and vitamin-enriched diet. Figure 7*A* demonstrates that addition of 40 mM KCl significantly increased glutamate release in tissue prepared from saline-injected rats fed on the control and experimental diets ($p < 0.05$; ANOVA). The KCl-induced enhancement of release was inhibited in tissue prepared from IL-1β-injected rats that were fed on the control diet, but this inhibition was reversed in rats that

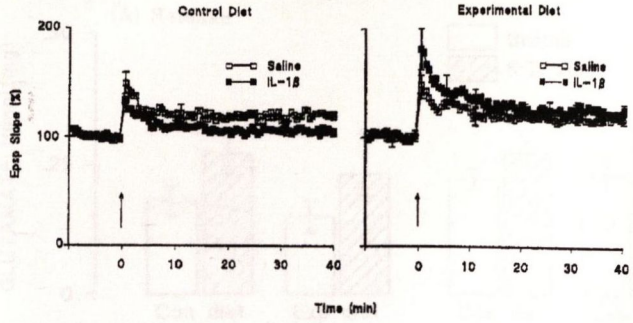

Figure 5. The IL-1β-induced impairment in LTP was reversed by dietary manipulation with antioxidant vitamins E and C. LTP was inhibited in IL-1β-injected rats fed on the control diet, but supplementation with a diet enriched in vitamins E and C (see Materials and Methods for details) reversed this effect. Dietary manipulation did not affect LTP in saline-treated rats. The data are derived from six observations in each treatment group, and SEM values are included for every 10th response.

received the experimental diet, such that KCl-stimulated release was significantly greater than unstimulated release ($p < 0.05$; ANOVA).

Analysis of the effect of these treatments on activity of JNK revealed a significant increase in enzyme activity in IL-1β-treated rats that were fed on the control diet compared with enzyme activity in saline-treated rats in either dietary group or IL-1β treated rats fed on the experimental diet ($p < 0.05$; Student's *t* test; Fig. 7*B*). In parallel with these effects on JNK activity, we observed that p38 activity was similar in tissue prepared from saline-pretreated rats fed on either diet, whereas enzyme activity was significantly increased in IL-1β-pretreated rats fed on the control diet ($p < 0.05$; Student's *t* test for paired values; Fig. 7*C*). Assessment of p38 activity was made in each of the four conditions (saline-pretreated rats and IL-1β-pretreated rats fed on control and experimental diets) on six separate samples and, in each case, enzyme activity was enhanced in tissue prepared from IL-1β-pretreated rats fed on control diet compared with saline-pretreated rats. Thus, dietary manipulation reversed the effects of IL-1β injection. Sample immunoblots indicate these trends in activities of JNK (Fig. 7*B*) and p38 (Fig. 7*C*).

DISCUSSION

We set out to investigate the downstream consequences of an increase in IL-1β in hippocampus, with the objective of increasing our understanding of the mechanisms that might contribute to impairment of LTP in dentate gyrus. The data indicate that among the cellular consequences of increased IL-1β concentration that accompany the impairment in LTP is an increase in reactive oxygen species production with the consequent increased activity of JNK and p38. On the basis of the evidence presented we propose that the IL-1β-induced increase in reactive oxygen species production increases activation of JNK and p38 which, in turn, inhibits LTP. IL-1β-induced inhibition of LTP may also result from the observed inhibition of glutamate release. This working hypothesis is presented in schematic form in Figure 8.

Consistent with this hypothesis, we report that the inhibitory effect of IL-1β on LTP in perforant path-granule cell synapses was accompanied by increased activity of both JNK and p38. The IL-1β-induced attenuation of LTP confirms our previous observations (Murray and Lynch, 1998a) and also confirms the results of *in vitro* experiments that demonstrated that IL-1β inhibited LTP in dentate gyrus (Cunningham et al., 1996), CA1 (Bellinger et al., 1993), and CA3 (Katsuki et al., 1990). Coupled with the IL-1β-induced inhibition of LTP, we observed an IL-1β-induced inhibition of KCl-induced glutamate release. Thus, whereas an increase in KCl-stimulated release was observed in synaptosomes prepared from tetanized tissue obtained from saline-treated rats, KCl-stimulated release was markedly decreased in synaptosomes prepared from both untetanized and tetanized tissue obtained from IL-1β-treated rats. These data support the hypothesis that LTP in dentate gyrus is tightly coupled with an increase in glutamate release at perforant path-granule cell synapses (Canevari et al., 1994; McGahon and Lynch, 1996; McGahon et al., 1999). We observed that IL-1β increased unstimulated release of glutamate, however this change did not always reach statistical significance.

We observed that the inhibitory effect of IL-1β on LTP was coupled with a stimulatory effect on the activities of JNK and p38. Thus, whereas activity of both kinases was unaffected by tetanic

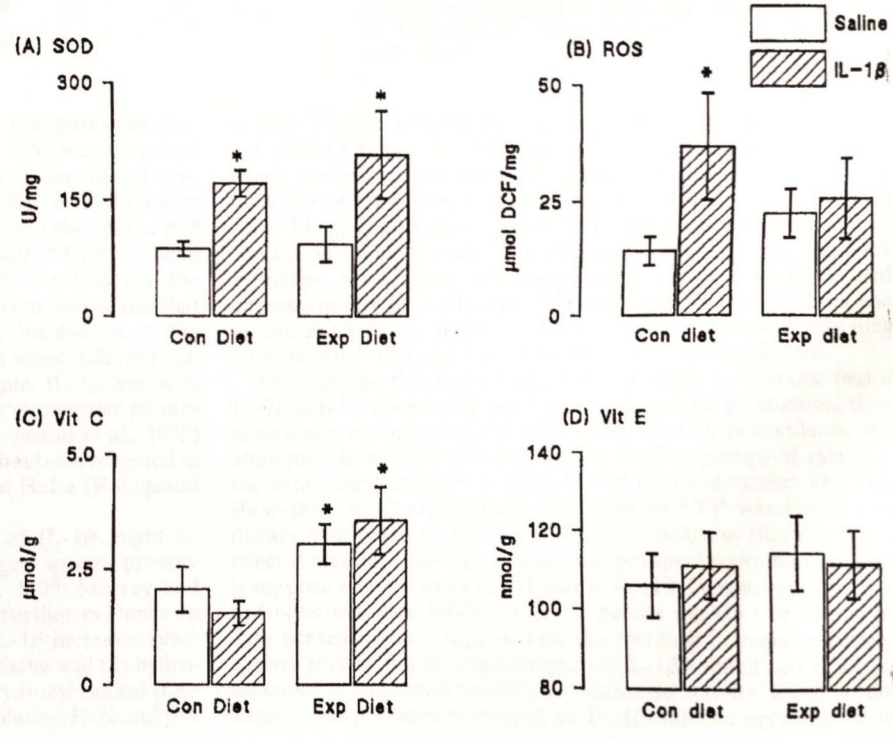

Figure 6. Dietary manipulation reversed the IL-1β-induced increase in reactive oxygen species formation. Intracerebroventricular injection of IL-1β significantly increased activity of superoxide dismutase (*SOD*; $p < 0.05$; Student's *t* test for paired means); dietary manipulation did not affect activity of the enzyme (*A*). Reactive oxygen species formation (*ROS*) was significantly increased in hippocampal synaptosomes prepared from IL-1β-injected rats compared with synaptosomes prepared from saline-injected rats (p 0.05; Student's *t* test for paired means). Dietary manipulation reversed this effect (*B*). The concentrations of vitamin C (*C*) and vitamin E (*D*) were similar in hippocampal tissue prepared from saline-injected and IL-1β-injected rats, but dietary manipulation significantly increased vitamin C concentration in tissue prepared from both groups of rats ($p < 0.05$; Student's *t* test for paired means). Vitamin E concentration was unaffected by diet. The values are means (± SEM) of six observations in each case.

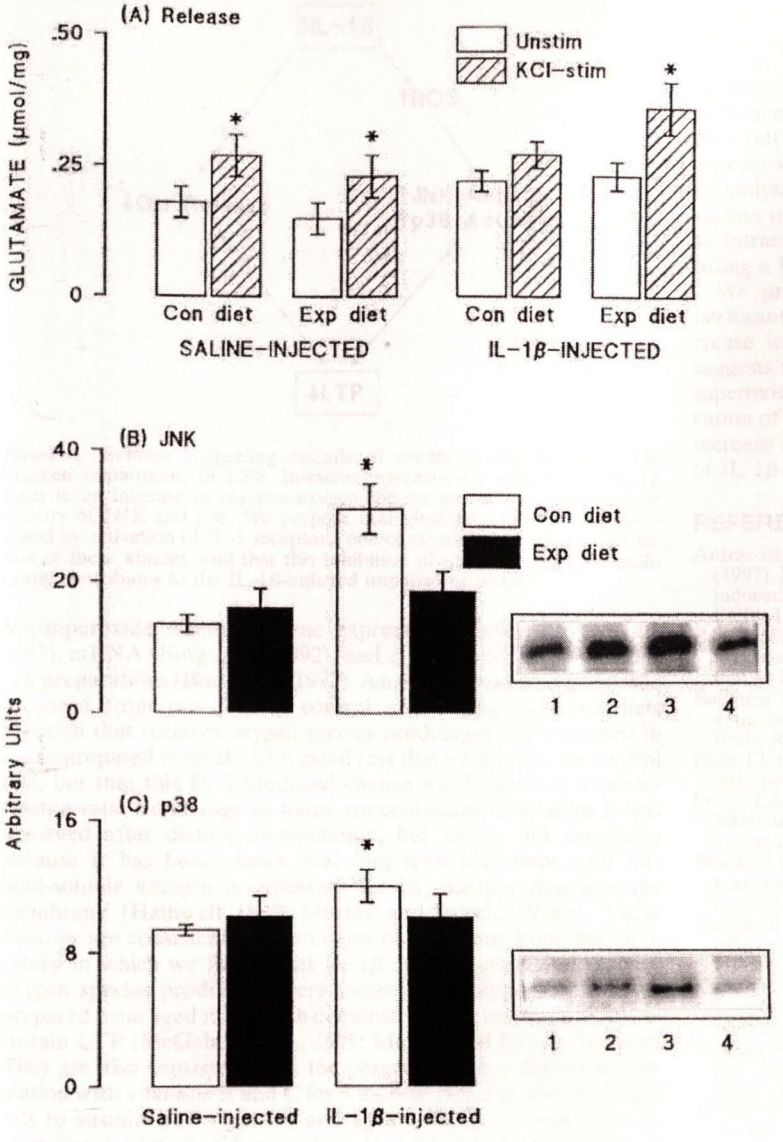

Figure 7. Dietary manipulation reversed some of the effects of IL-1β. *A,* Glutamate release was significantly increased by addition of 40 mM KCl to synaptosomes of dentate gyrus prepared from saline-injected rats fed on both the control and experimental diets (*$p < 0.05$; Student's *t* test for paired values). This effect was blocked in synaptosomes prepared from dentate gyrus of IL-1β-injected rats that were fed on the control diet, but the attenuation was reversed in synaptosomes prepared from IL-1β-injected rats that were fed on the experimental diet (*$p < 0.05$; Student's *t* test for paired values). Activities of JNK (*B*) and p38 (*C*) were assessed in aliquots of hippocampal synaptosomes prepared from the same rats. Dietary manipulation did not significantly alter activity of either enzyme in samples prepared from saline-injected rats. However, activities of both JNK and p38 were significantly increased in synaptosomes of IL-1β-injected rats (compare *lanes 3* and *4* with *lanes 1* and *2*), as shown by the sample immunoblots and mean data derived from densitometric analysis; (*$p < 0.05$; Student's *t* test for paired values). The IL-1β-induced changes in activities of both enzymes were blocked by dietary manipulation (*lanes 2* and *4*). The values are means (± SEM) of six observations in all experiments.

stimulation, both were markedly increased in synaptosomes prepared from hippocampus obtained from rats that were injected with IL-1β. One interpretation of this finding is that IL-1β activates these kinases and that this action may result in inhibition of LTP; this is consistent with the reported inhibitory effect of the p38 inhibitor SB203580 on IL-1β-induced attenuation of LTP (Coogan et al., 1997). To address this question directly, we analyzed the effect of IL-1β on activity of JNK and p38 *in vitro* and found that activities of both kinases were increased by incubation in the presence of the cytokine. A number of groups using different cell types have reported such an action. For example, IL-1β has been reported to increase activity of JNK in human glomerular mesangial (Uciechowski et al., 1996) and HeLa (Raingeaud et al., 1995) cells, whereas IL-1β-induced activation of p38 has been reported in Chinese hamster CCl39 (Guay et al., 1997) and HeLa (Raingeaud et al., 1995) cells.

It has been suggested that certain effects of IL-1β might be mediated through an increase in reactive oxygen species production (Sumoski et al., 1989; Raingeaud et al., 1995; Murray and Lynch, 1998b), and in this study we provide further evidence to support this view by demonstrating that (1) IL-1β increases reactive oxygen species formation in hippocampal tissue and (2) hydrogen peroxide, which leads to formation of the hydroxyl radical (Qin et al., 1999), mimics the effect of IL-1β by stimulating JNK and p38 *in vitro*. These findings are consistent with the observation that UV radiation (Zhang et al., 1997) and osmotic stress (Qin et al., 1999), which also induce formation of reactive oxygen species, stimulate activities of both JNK and p38 (Raingeaud et al., 1995) in HeLa cells. These results support our working hypothesis; if IL-1β concentration in hippocampus is increased, reactive oxygen species formation is increased, stress-activated kinases are activated, and expression of LTP is blocked. Although the data, in particular the *in vitro* analyses, are consistent with the idea that these effects may be sequential, this remains to be unequivocally established.

To challenge this hypothesis, it is reasonable to propose that if IL-1β acts by stimulating reactive oxygen species production, then subsequent actions of IL-1β will be inhibited by antioxidants. We attempted to address this question by feeding groups of rats with the antioxidant vitamins E and C before IL-1β injection. The data show that the IL-1β-induced inhibition of LTP was blocked by dietary manipulation, leading us to the conclusion that the IL-1β effect is mediated through reactive oxygen species production. This is supported by the work of Pellmar et al. (1991), who reported that hydrogen peroxide inhibits LTP in guinea pig CA1 *in vitro*. The data presented here suggest that the increase in reactive oxygen species production in hippocampus of IL-1β-treated rats is a consequence of increased superoxide dismutase activity, which is consistent with previous reports of an IL-1β-induced upregulation of

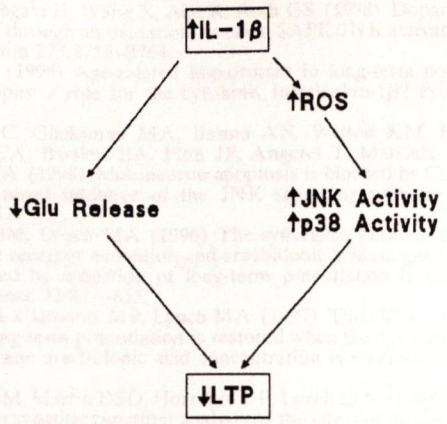

Figure 8. Scheme suggesting cascade of events leading to the IL-1β-induced impairment in LTP. Intracerebroventricular injection of IL-1β leads to an increase in reactive oxygen species production that increases activity of JNK and p38. We propose that glutamate release is compromised by activation of IL-1 receptors, one consequence of which is activation of these kinases, and that this inhibition of glutamate release significantly contributes to the IL-1β-induced impairment in LTP.

Mn-superoxide dismutase gene expression (Antras-Ferry et al., 1997), mRNA (Borg et al., 1992), and enzyme activities in various cell preparations (Borg et al., 1992). Analysis of hippocampal tissue prepared from rats fed on control and vitamin-enriched diets revealed that reactive oxygen species production was increased in tissue prepared from IL-1β-treated rats that were given the control diet, but that this IL-1β-induced change was blocked in vitamin-treated rats. No change in tissue concentration of vitamin E was observed after dietary manipulation, but this is not surprising because it has been shown that long-term treatment with this lipid-soluble vitamin is necessary for its incorporation into the membrane (Halliwell 1992; Murray and Lynch, 1998b). These findings are consistent with previous observations from this laboratory in which we found that IL-1β concentration and reactive oxygen species production were increased in hippocampal tissue prepared from aged rats, which demonstrated an impaired ability to sustain LTP (McGahon et al., 1997; Murray and Lynch, 1998a,b). They are also consistent with the observation that dietary manipulation with vitamins E and C for 3 months restored ability of aged rats to sustain LTP (Murray and Lynch, 1998b). Predictably, dietary manipulation did not effect IL-1β-induced stimulation of superoxide dismutase, therefore we propose that the enhanced tissue concentration of vitamin C that was observed after dietary manipulation was responsible for reversing the IL-1β-induced increase in reactive oxygen species production.

To obtain further evidence in support of the hypothesis that antioxidant treatment reverses the effects of IL-1β on hippocampal function, we analyzed glutamate release and activity of JNK and p38 in synaptosomes prepared from saline-treated and IL-1β-treated rats fed on the control and vitamin-enriched diets. The data showed that the inhibitory effect of IL-1β on release was blocked by dietary manipulation. Coupled with this change, we observed that dietary manipulation reversed the IL-1β-induced increases in activities of JNK and p38. These results suggest that the increase in tissue vitamin C induced by dietary manipulation prevents the stimulatory effect of IL-1β and its subsequent inhibitory effect on glutamate release. If the argument that the inhibitory effect of IL-1β on LTP is a consequence of activation of JNK and/or p38, then it must be predicted that LTP will be inhibited, and activities of JNK and/or p38 will be increased when endogenous IL-1β concentration in hippocampus is increased, for example in hippocampal tissue prepared from aged rats (Murray and Lynch, 1998a,b). We have recently observed that JNK activity and p38 activity are increased in hippocampal tissue prepared from aged rats (O'Donnell et al., 2000), in which IL-1β concentration and reactive oxygen species production are increased (Lynch, 1998).

Increased activation of both JNK and p38 have been associated with cell death (Yang et al., 1997; Luo et al., 1998), and evidence suggests that IL-1β plays a role in cell death induced by ischemia (Rothwell, 1999). It might be speculated that, under the current experimental conditions, IL-1β stimulates JNK and/or p38 and that cell damage might result from this action. This proposal concurs with our recent finding that parenteral administration of lipopolysaccharide increased IL-1β concentration and cell degeneration in entorhinal cortex and that these changes were inhibited by intracerebroventricular injection of a caspase-1 inhibitor indicating a key role for IL-1β (Campbell et al., 2000).

We propose that the inhibitory effect of IL-1β on LTP in perforant path-granule cell synapses is a consequence of an increase in reactive oxygen species formation, and the evidence suggests that the primary effect of IL-1β is to stimulate activity of superoxide dismutase. Our evidence suggests that increased activation of JNK and p38 represent critical downstream events of the increase in reactive oxygen species, leading to the inhibitory effect of IL-1β.

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Age-related impairment in LTP is accompanied by enhanced activity of stress-activated protein kinases: analysis of underlying mechanisms

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Abstract

The age-related impairment in long-term potentiation in the rat dentate gyrus is coupled with an increase in the proinflammatory cytokine, interleukin-1β (IL-1β). It is possible that this increase in IL-1β might be a consequence of the age-related increase in reactive oxygen species production in hippocampal tissue. In this study we set out to identify the underlying cause of the age-related increase in reactive oxygen species production and to establish whether any consequences of such a change might impact on the ability of aged rats to sustain long-term potentiation (LTP). We report that there was an age-related increase in the activity of superoxide dismutase but no parallel increases in activities of glutathione peroxidase or catalase, while age-related decreases in the concentration of the scavengers, vitamins E and C and glutathione were also observed. We propose that these compromises in antioxidative strategies may result in an increase in reactive oxygen species production. The data described indicate that IL-1β and H_2O_2 increase the activity of two stress-activated mitogen-activated protein kinases, c-Jun NH_2-terminal kinase (JNK) and p38 *in vitro*, while age-related increases in both kinases were observed. We propose that the endogenous increase in these parameters which occurs with age induces the increase in activity of the stress-activated kinases, which in turn impacts on the ability of the aged rat to sustain LTP.

Introduction

In rats, one effect of age is an impairment in cognitive function (Barnes, 1979; 1988), and this is accompanied by an impaired ability to sustain long-term potentiation (LTP, Landfield *et al.*, 1978; Barnes, 1979; de Toledo-Morrell & Morrell, 1985; Lynch & Voss, 1994; McGahon *et al.*, 1997; Murray & Lynch, 1998a,b). We have recently reported that the impairment in LTP in the dentate gyrus of aged rats is accompanied by an increase in the concentration of the proinflammatory cytokine, interleukin-1β (IL-1β, Murray & Lynch, 1998a,b; Lynch, 1998). Further evidence which suggests a correlation between increased IL-1β concentration and compromised LTP are the findings that: (i) IL-1β inhibits LTP *in vitro* (Bellinger *et al.*, 1993; Cunningham *et al.*, 1996) and *in vivo* (Lynch, 1998; Murray & Lynch, 1998a); and (ii) IL-1β concentration is increased in the dentate gyrus of stressed rats which demonstrate a poor ability to sustain LTP (Murray & Lynch, 1998a).

Our evidence suggests that the impairment in LTP and the increase in IL-1β concentration are also associated with an age-related increase in reactive oxygen species production (Cunningham *et al.*, 1996; Murray & Lynch, 1998a). It has been known for decades that several properties of neuronal tissue confer on it a particular susceptibility to oxidative insult. Among these properties are a high oxygen consumption, modest antioxidant defences (Halliwell, 1992), a high concentration of iron (Benkovic & Connor, 1993) and high concentrations of polyunsaturated fatty acids, which are known to generate oxygen radicals following oxidation (Rice-Evans & Burdon, 1993). Protection against reactive oxygen species relies on enzymatic and non-enzymatic strategies; compromises in both have been reported in the aged cortex (O'Donnell & Lynch, 1998). There is some evidence that antioxidant strategies are also compromised in the aged hippocampus. For example, we have observed a decrease in the concentration of vitamin E (Murray & Lynch, 1998b), while an increase in formation of hydroxyl ions has been reported in the hippocampus of the aged gerbil (Zhang *et al.*, 1993).

A number of cellular responses are shared by IL-1β and reactive oxygen species; in particular, both have been shown to activate certain mitogen-activated protein (MAP) kinases which are implicated in cell death. The members of the MAP kinase family fall into three groups, extracellular signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK) or stress-activated protein kinase (SAPK) and p38. While ERK is stimulated by growth factors and activation results in neurite outgrowth, cell proliferation or differentiation (e.g. Seger & Krebs, 1995; Xia *et al.*, 1995; Creedon *et al.*, 1996), JNK and p38 are activated by environmental stress including oxidative stress (Davis, 1995; Raingeaud *et al.*, 1995; Uciechowski *et al.*, 1996; Junger *et al.*, 1997), and activation leads to growth arrest or even cell death (Park *et al.*, 1996; Maroney *et al.*, 1998). There is a good deal of evidence which indicates that both JNK and p38 are also stimulated by IL-1β (Derijard *et al.*, 1994; Raingeaud *et al.*, 1995; Rizzo & Carlo-Stella, 1996; Uciechowski *et al.*, 1996; Lu *et al.*, 1997). Much of this evidence has been obtained in circulating cells

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and cultured cells with a dearth of evidence for similar changes in neuronal tissue.

In this study, we set out to assess the age-related changes in activities of JNK and p38 which accompany the impairment in LTP, and to establish whether any changes might be coupled with evidence of a compromise in antioxidative defences in the aged hippocampus. We demonstrate that the age-related impairment in LTP was associated with increased activity of both MAP kinases, and the present data are consistent with the hypothesis that these changes might be secondary to increased production of reactive oxygen species.

Materials and methods

Animals

Male Wistar rats (Charles River Laboratories, UK) of mean age 4 months (250–350 g) or 22 months (450–550 g) were used in these experiments. Animals were housed in pairs (22-month-old rats) or groups of four to six (4-month-old rats) under a 12 h light schedule. Ambient temperature was controlled between 22 and 23 °C. A total of seven young and 13 aged rats were used in these studies; hippocampal tissue obtained from these rats was used for subsequent biochemical analysis. Rats were maintained under veterinary supervision for the duration of the experiment and monitored daily. These experiments were performed under a license issued by the Department of Health (Ireland).

Induction of LTP in vivo

Rats were anaesthetized by intraperitoneal injection of urethane (1.5 g/kg i.p.). In the case of aged rats, 75% of the urethane dose was administered initially and topped up if necessary to induce an adequate level of anaesthesia, which was recognized to be the absence of a pedal reflex. Rats were placed in a head holder in a stereotaxic frame, and a window of skull was removed to allow placement of a bipolar stimulating electrode in the perforant path (4.4 mm lateral to lambda) and a unipolar recording electrode in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to Bregma). Test shocks (average stimulus strength 4.14 ± 0.15 V and 3.96 ± 0.12 V in young and aged rats, respectively) were given at 30 s intervals for 10 min before and 40 min after tetanic stimulation (three trains of stimuli; 250 Hz for 200 ms; 30 s intertrain interval). This protocol has been shown to induce saturated LTP in perforant path-granule cell synapses in anaesthetized rats (Williams *et al.*, 1989), and has been shown to induce LTP in some, but not all, aged rats (Lynch & Voss, 1994). In a separate experiment, aged and young rats were divided into two groups. One subgroup of young rats and one subgroup of aged rats were fed for 8 weeks on an experimental diet of laboratory chow supplemented with a daily dose of 10 mg docosahexanoic acid (26% w/v in tuna oil; Laxdale Research, UK). The other subgroups received standard laboratory chow to which corn oil was added; in this way the different groups of rats received an isocaloric intake. Food intake was measured for 2 weeks before the commencement of the experiment. Sufficient diet was prepared for 3 or 4 days at a time and daily allowances were stored in airtight containers at –80 °C. In this study, data are reported from 10 young rats (six fed on control and four fed on experimental diet) and 14 aged rats (six fed on control and eight fed on experimental diet). Data from larger groups of aged and young rats, fed on control and experimental diets, have been reported elsewhere (McGahon *et al.*, 1999b). The availability of tissue was the only factor determining the numbers of rats used for the present analyses.

At the end of the recording period, rats were killed by cervical dislocation, the hippocampi were removed and chopped twice, the second time at an angle of 45°, to provide tissue prisr (350 × 350 μm). Tissue was frozen in Krebs solution containi 10% dimethylsulphoxide (DMSO, Haan & Bowen, 1981) and stor at –80 °C until required. IL-1β and vitamin E concentrations, as w as activities of superoxide dismutase, catalase and glutathio peroxidase, were assessed in homogenate prepared from hippocamp slices while, at the time of dissection, a portion of the hippocamp tissue was homogenized in Krebs solution containing 5% trichlo oacetic acid (TCA) for later analysis of ascorbic acid and glutathion Reactive oxygen species production and activities of JNK and p. were assessed in the crude synaptosomal pellet, P_2.

Analysis of the activity of the MAP kinases

Samples were analysed for protein (Bradford, 1976), diluted to equ protein concentration, and aliquots (10 μL, 1 mg/mL) were added 10 μL sample buffer [Tris–HCl, 0.5 mM, pH 6.8; glycerol, 10% sodium dodecyl sulphate (SDS), 10%; β-mercaptoethanol, 5% bromophenol blue, 0.05% w/v], boiled for 5 min and loaded ont gels (10% SDS for p38, and 12% for JNK). Proteins were separate by application of 30 mA constant current for 25–30 min, transferre onto nitrocellulose strips (225 mA for 75 min) and immunoblotte with the appropriate antibody. To assess JNK activity, proteins wer immunoblotted with an antibody which specifically targets pho phorylated JNK [Santa Cruz, USA; 1 : 2000 in phosphate-buffere saline (PBS)–Tween (0.1% Tween-20) containing 2% non-fat drie milk] for 2 h at room temperature. Nitrocellulose strips were washe and incubated for 2 h at room temperature with secondary antibod (peroxidase-linked antimouse IgG; 1 : 2000 dilution; Sigma, UK). T assess p38 activity, proteins were immunoblotted with an antibod which specifically targets phosphorylated p38 [Santa Cruz; 1 : 1000 PBS–Tween (0.1% Tween-20) containing 2% non-fat dried milk] f 2 h at room temperature. Nitrocellulose strips were washed an incubated for 2 h at room temperature with secondary antibod (peroxidase-linked antimouse IgM; 1 : 1000 dilution; Sigma). Protei complexes were visualized by ECL detection (Amersham, UK Immunoblots were exposed to film overnight, and processed using Fuji X-ray processor.

The effects of H_2O_2 and IL-1β were assessed on activities of JN and p38. Synaptosomes prepared from the hippocampus we incubated for 15 min at 37 °C in Krebs solution in the presence absence of H_2O_2 (5 mM), or in the presence or absence of IL-1 (10 pg/mL), and the analysis proceeded as described above.

Analysis of reactive oxygen species formation

The formation of reactive oxygen species was assessed hippocampal tissue according to the method of Lebel & Bonc (1990). It employs the non-fluorescent probe, 2′7′-dichlorofluoresce diacetate (DCFH-DA; Molecular Probes, USA), which after d esterification is oxidized, in the presence of reactive oxygen speci to the highly fluorescent 2′,7-dichlorofluorescein (DCF). To asse reactive oxygen species production, the synaptosomal pellet, P_2, w resuspended in 1 mL ice-cold 40 mM Tris buffer (pH 7.4). Aliquots synaptosomes (1 mL) were incubated with DCFH-DA (10 μL; fin concentration 5 μM; from a stock solution of 500 μM in methanol) 37 °C for 15 min. In some experiments the effect of IL-1β on reacti oxygen species production was assessed by incubating tissue f 15 min at 37 °C in the presence of the cytokine. To terminate t reaction, the dye-loaded synaptosomes were centrifuged at 13 00 for 8 min. The pellet was resuspended in 3 mL ice-cold 40 mM T buffer, pH 7.4. Fluorescence was monitored at a constant temperatu of 37 °C immediately before stimulation with IL-1β (1 ng/mL), a 15 min post-stimulation, at 488 nm excitation (band width 5 nm) a

525 nm emission (band width 20 nm). Reactive oxygen species formation was quantified from a standard curve of DCF in methanol (range 0.05–1 μM). Protein concentration was determined (Bradford, 1976) and the results were expressed as nmol/mg protein/min.

Analysis of IL-1β expression

IL-1β concentration was assessed in homogenate prepared from hippocampus using an ELISA as previously described (Murray & Lynch, 1998a,b). In some experiments, to assess the effect of H_2O_2 on IL-1β concentration, tissue was incubated for 15 min at 37 °C in the presence or absence of H_2O_2 (5 mM). Slices were then homogenized and equalized for protein for analysis. Ninety-six-well plates were coated with capture antibody (monoclonal hamster antimouse IL-1β antibody; Genzyme Diagnostics, USA), and aliquots (100 μL) of samples or IL-1β standards (0–1000 pg/mL) were added and incubated. Secondary antibody (100 μL; final concentration 0.8 μg/mL in PBS; biotinylated polyclonal rabbit antimouse IL-1β antibody) was added, plates were incubated and washed, and detection agent (100 μL horseradish peroxidase-conjugated streptavidin; 1 : 1000 dilution in PBS containing 0.05% Tween 20 and 1% bovine serum albumin) was then added. Samples were incubated with tetramethylbenzidine liquid substrate (100 μL; Sigma) and absorbance was read at 450 nm.

Analysis of superoxide dismutase activity

Superoxide dismutase activity was determined according to the method described by Spitz & Oberley (1989). Aliquots (800 μL) of incubation buffer [50 mM potassium buffer (pH 7.8) containing (in mM): xanthine, 1.8; nitroblue tetrazolium (NBT), 2.24; catalase, 40 units; xanthine oxidase, 7 μL/mL; diethylenetriaminepentacetic acid, 1.33] were added to 1.5-mL microfuge tubes containing samples of supernatant (100 μL) at different dilutions (1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 50 and 1 : 100) and analysed by UV spectroscopy at 560 nm. In some experiments, slices were incubated for 30 min at 37 °C in the presence of IL-1β (3.5 ng/mL) to analyse the effect of the cytokine on superoxide dismutase activity. Slices were homogenized and enzyme activity was assessed as the rate of reduction of NBT, which was inhibited with increasing concentrations of protein. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%.

Analysis of glutathione peroxidase activity

Glutathione peroxidase activity was measured according to the method of Lawrence & Burk (1976). Samples of supernatant (100 μL) were added to incubation buffer [50 mM potassium phosphate (pH 7), containing (in mM): EDTA, 1; sodium azide, 1; NADPH, 0.2; glutathione reductase, 1 unit; GSH glutathione, 1; cumene hydroperoxide, 1.5; 800 μL] and incubated for 5 min at room temperature. The reaction was initiated by addition of cumene hydroperoxide (15 mM; 100 μL). Absorbance was recorded at 340 nm for 5 min; activity was calculated from the slope (i.e. change in absorbance with time).

Analysis of catalase activity

Catalase activity was determined as previously described (Cohen *et al.*, 1996). Briefly, aliquots of supernatant (50 μL) were added to microfuge tubes containing buffer (10 mM potassium phosphate, pH 7.0; 800 μL) and kept on ice. The reaction was initiated by the addition of ice-cold H_2O_2 (60 mM; 100 μL), samples were mixed and incubated on ice for 2 or 10 min, after which time aliquots (100 μL) were removed and quenched by addition to H_2SO_4 (0.6 N; 4 mL) and $FeSO_4$ (10 mM; 1 mL) at room temperature. Colour was developed at room temperature by addition of 2.5 M potassium thiocyanate (400 μL). Aliquots (200 μL) were transferred immediately to 96-well plates, absorbance was read at 492 nm, and results were expressed in terms of the first order reaction rate constant (k), corrected for protein using the formula: enzyme units = k/protein = [ln ($A1$/$A2$/t]/protein, where $A1$ and $A2$ represented the absorbance at the two selected time points (i.e. 2 and 10 min), and t represents the time difference between the two points (i.e. 8 min).

Analysis of ascorbic acid

Ascorbic acid concentrations were determined as previously described (Omaye *et al.*, 1979). Briefly, duplicate aliquots of supernatant (100 μL) were added to a 2,4-dinitrophenylhydrazine/thiourea/copper (DTC) solution (in mM: thiourea, 50; copper sulphate, 2; dinitrophenylhydrazine, 150; in 9 N H_2SO_4; 20 μL) and incubated for 3 h at 37 °C. Ice-cold H_2SO_4 (65%; 150 μL) was added to stop the reaction, samples were vortex-mixed and incubated at room temperature for 30 min before aliquots (100 μL) were transferred to 96-well plates for assessment by UV spectroscopy at 545 nm. Ascorbic acid standards were prepared in 5% TCA.

Analysis of α-tocopherol

α-Tocopherol was analysed according to the method of Vatassery (1994). Briefly, aliquots of homogenate (150 μL) were incubated in the presence of ethanol containing 0.025% butyl-hydroxytoluene (150 μL), 25% ascorbic acid (70 μL) and 10% potassium hydroxide (135 μL) for 30 min at 60 °C. Hexane (540 μL) containing 0.025% butyl-hydroxytoluene was added, samples were vortex-mixed for 1 min and centrifuged at 1500 r.p.m. for 6 min. The hexane phase was removed and evaporated to dryness under nitrogen; the recovery of α-tocopherol using this procedure was between 70 and 80%. For high-performance liquid chromatography (HPLC) analysis, dried samples were resuspended in methanol (150 μL; Romil, UK) containing 0.025% butyl-hydroxytoluene, and 30-μL volumes were injected onto an Intersil C18 column (maintained at 40 °C; Alltech, USA). Separation of α-tocopherol was achieved using a mobile phase of 75% acetonitrile : 25% methanol at a flow rate of 1.2 mL/min, and samples were detected by UV spectroscopy at 292 nm. α-Tocopherol concentration was estimated by the external standard method and expressed as μg/mg tissue.

Analysis of glutathione

Total glutathione was measured as described previously (Tietze, 1969). Briefly, aliquots of supernatant (30 μL) prepared from homogenate were incubated for 1 min on ice with sample buffer (100 mM potassium phosphate containing 5 mM EDTA, pH 7.5; 450 μL), in the presence of glutathione reductase (100 μL; 5 units/mL) and 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (50 μL). NADPH (2.4 mM; 100 μL) was added and the absorbance monitored for 2 min at 412 nm. Except where indicated, all reagents were purchased from Sigma (UK).

Results

We compared the ability of aged and young rats to sustain LTP, and in these experiments, aged rats exhibited an attenuated response to tetanic stimulation (Fig. 1A). The mean percentage changes in excitatory post-synaptic potential (EPSP) slope in the 2 min immediately following tetanic stimulation (compared with the 5 min value immediately prior to stimulation) were 153.02 (± 3.85) and 116.45 (± 2.72) in the 4-and 22-month-old rats, respectively. The mean percentage changes in EPSP slope in the last 5 min of the

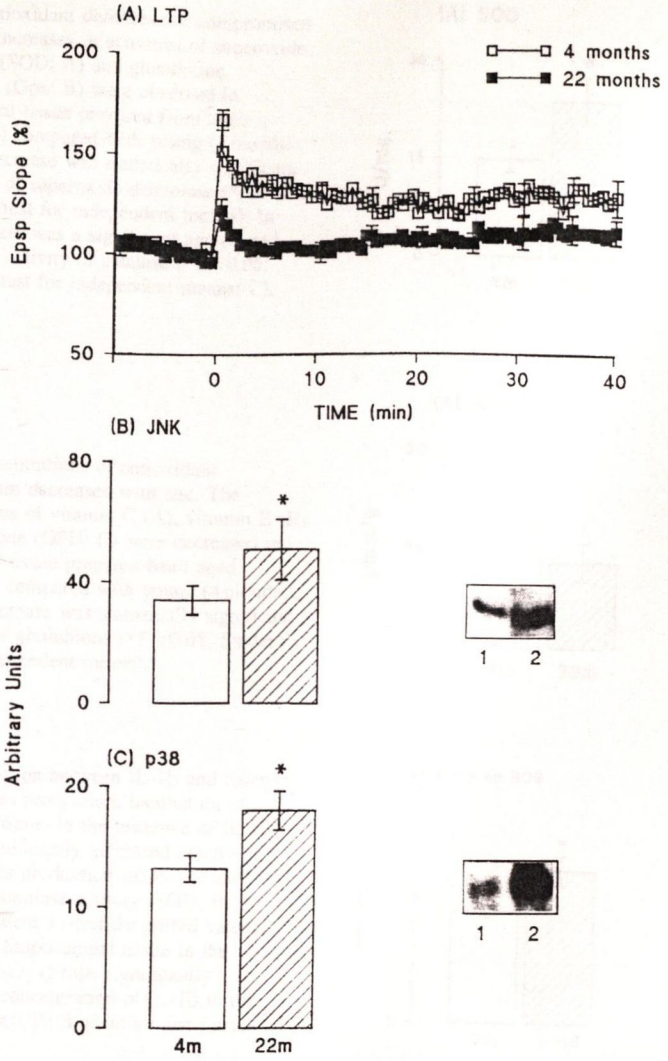


FIG. 1. Ageing is associated with a decrease in LTP and an increase in activities of JNK and p38. (A) Tetanic stimulation induces an immediate increase in EPSP slope in both aged and young rats, but the ability of aged rats to sustain LTP is impaired compared with young rats. Activities of JNK (B) and p38 (C) were significantly increased in synaptosomes prepared from the hippocampus of aged rats, compared with young rats (*$P<0.05$; Student's *t*-test for independent means; $n=6$). Sample immunoblots illustrating these changes are shown.

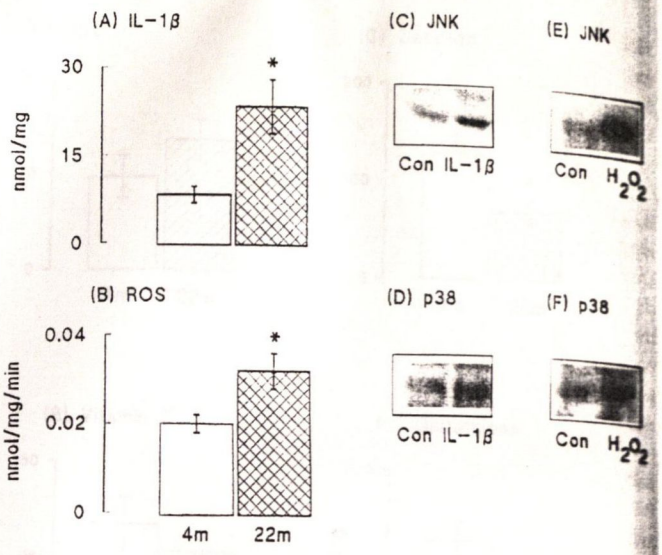


FIG. 2. Increases in IL-1β concentration and reactive oxygen species production *in vivo* might underlie the age-related increases in activities of JNK and p38. Increases in IL-1β concentration (A) and reactive oxygen species production (B) were observed in hippocampal synaptosomes prepared from aged (22 months), compared with young (4 months), rats (*$P<0.05$; Student's *t*-test for independent means). Incubation of hippocampal synaptosomes in the presence of IL-1β (10 pg/mL) increased activities of JNK (C) and p38 (D). Similarly, incubation of hippocampal synaptosomes in the presence of H_2O_2 (5 mM) increased activities of both JNK (E) and p38 (F).

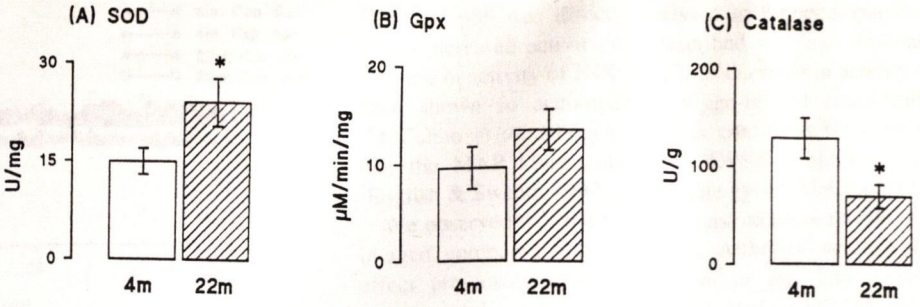
experiment were 136.45 (± 2.15) and 110.35 (± 2.08). These data indicate that there was a significant age-related decrease in both the early and persistent response to tetanic stimulation ($P<0.05$; Student's *t*-test for unpaired samples).

We assessed the activity of JNK and p38 in tissue prepared from the hippocampus obtained from young and aged rats using phosphospecific antibodies. One sample immunoblot indicating an age-related increase in JNK activity is shown in Fig. 1B. JNK activity was quantified by densitometry, and the mean data obtained from analysis of six similar experiments indicate a significant age-related increase ($P<0.05$; Student's *t*-test for independent means; Fig. 1B). Activity of p38 was also increased with age as demonstrated by a sample immunoblot (Fig. 1C). Analysis of the mean data obtained from densitometric analysis indicated that the age-related increase in kinase activity reached statistical significance ($P<0.05$; Student's *t*-test for independent means; $n=6$).

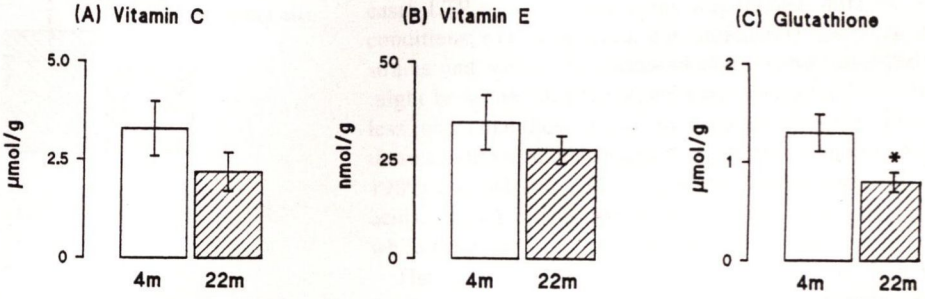
In an effort to establish the age-related changes which might impact on JNK and p38 activity, we compared IL-1β concentration and reactive oxygen species production in hippocampal tissue prepared from aged and young rats, and analysed the effect of IL-1β and H_2O_2 on activities of both kinases. Figure 2A shows that the concentration of IL-1β was significantly increased in hippocampal tissue prepared from aged rats compared with young rats ($P<0.05$; Student's *t*-test for unpaired samples). Similarly, we observed that there was an age-related increase in reactive oxygen species production ($P<0.05$; Student's *t*-test for unpaired samples; Fig. 2B). We observed that IL-1β and H_2O_2 both increased activities of JNK (Fig. 2C and E) and p38 (Fig. 2D and F).

We considered that the age-related increase in reactive oxygen species production might be a consequence of a compromise in antioxidative defence mechanisms; we therefore assessed age-related changes in enzymatic and non-enzymatic antioxidative defences. Figure 3A indicates that superoxide dismutase activity was significantly increased in hippocampal tissue prepared from aged rats compared with tissue prepared from young rats ($P<0.05$; Student's *t*-test for independent data; $n=8$). There was no evidence of any age-related change in glutathione peroxidase activity (Fig. 3B; $n=8$), but activity of catalase was decreased in tissue prepared from the hippocampus of aged, compared with young, rats ($P<0.05$; Student's *t*-test for independent data; Fig. 3C; $n=8$). The data also demonstrate that there were age-related decreases in the concentrations of vitamin E, vitamin C and glutathione ($n=8$ in each case). Figure 4 indicates that while this difference was not statistically significant in the case of vitamin E (Fig. 4B) or vitamin C (Fig. 4A), the aged-related decrease in glutathione (Fig. 4C) was statistically significant ($P<0.05$; Student's *t*-test for independent data).

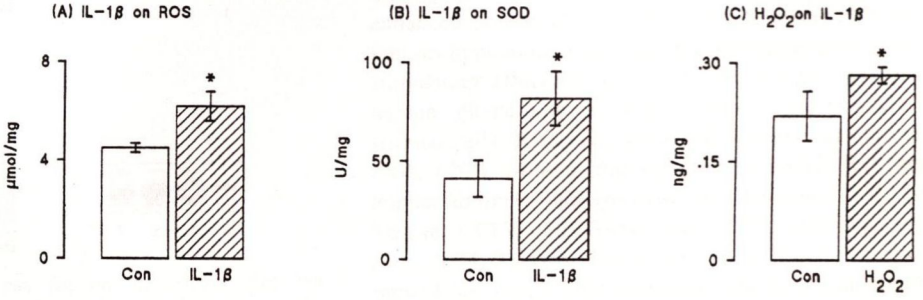
To probe the interaction between IL-1β and reactive oxygen species production, we analysed the effect of IL-1β on reactive oxygen species production and superoxide dismutase activity, and the effect of H_2O_2 on IL-1β concentration. Figure 5A and B shows that IL-1β significantly increased reactive oxygen species production and superoxide dismutase activity ($P<0.05$; Student's *t*-test for paired



3. Antioxidant defences are compromised h age. Increases in activities of superoxide mutase (SOD; A) and glutathione oxidase (Gpx; B) were observed in pocampal tissue prepared from aged months) compared with young (4 months) s. The increase was statistically significant he case of superoxide dismutase ($*P<0.05$; dent's *t*-test for independent means). In ntrast, there was a significant age-related rease in activity of catalase ($*P<0.05$; dent's *t*-test for independent means; C).



4. Concentrations of antioxidant vengers are decreased with age. The centrations of vitamin C (A), vitamin E (B) glutathione (GSH; C) were decreased in pocampal tissue prepared from aged months), compared with young (4 months), s. This decrease was statistically significant he case of glutathione ($*P<0.05$; Student's st for independent means).



5. Interaction between IL-1β and reactive gen species production. Incubation of pocampal tissue in the presence of IL-1β g/mL) significantly increased reactive gen species production (ROS; A) and eroxide dismutase activity (SOD; B; <0.05; Student's *t*-test for paired values). ubation of hippocampal tissue in the sence of H_2O_2 (5 mM) significantly eased the concentration of IL-1β in the ue (C; $*P<0.05$; Student's *t*-test for paired ues).

nples; $n=6$), while H_2O_2 significantly increased IL-1β concentra- n ($P<0.05$; Student's *t*-test for paired samples; Fig. 5C; $n=6$).

To strengthen the coupling between LTP, IL-1β concentration and ivities of JNK and p38, we assessed the ability of aged ($n=14$) and ng ($n=10$) rats, fed on control (six young and six aged rats) and erimental (four young and eight aged rats) diets, to sustain LTP. pocampal tissue prepared from these rats was assessed for IL-1β centration and enzyme activities. Figure 6A shows that dietary nipulation did not affect LTP in young rats. In contrast, LTP was mpromised in aged rats fed on the control diet, but this impairment s reversed in aged rats fed on the experimental diet. IL-1β centration in hippocampal tissue was similar in both groups of ng rats, but was significantly increased in tissue prepared from d rats which were fed on the control diet (Fig. 6B; $P<0.001$; dent's *t*-test for independent means). Dietary manipulation ersed the age-related increase in IL-1β concentration. Tissue pared from these rats was also assessed for activities of JNK . 6C) and p38 (Fig. 6D). The sample immunoblots and the mean (derived from densitometric analysis) indicate age-related eases in enzyme activities in tissue prepared from rats fed on control diet compared with young rats fed on the control diet 0.05; Student's *t*-test for independent means). Dietary manipula- in young rats did not significantly affect enzyme activity, though it increased variability as reflected by the increase in standard error; however, it reversed the age-related increases in enzyme activities.

Discussion

The objective of this study was to identify the changes responsible for the age-related increase in reactive oxygen species production in the rat hippocampus and to analyse some of the downstream consequences of these changes which might impact on the age-related impairment in LTP. We report an age-related compromise in LTP and an age-related increase in activity of the two MAP kinases, JNK and p38. We present data which indicate that activities of both kinases are increased by IL-1β and H_2O_2 *in vitro*. The results presented are consistent with the view that the age-related increase in reactive oxygen species production results from a compromise in antioxidant defence mechanisms.

In this study we observed that age was associated with an impairment in LTP in perforant path-granule cell synapses and increases in the activity of both JNK and p38. The impairment in LTP in aged rats has been reported many times previously (Landfield *et al.*, 1978; Barnes, 1979; de Toledo-Morrell & Morrell, 1985; Lynch & Voss, 1994; McGahon *et al.*, 1997; Murray & Lynch, 1998a, b), but, to our knowledge this is the first study in which the activity of

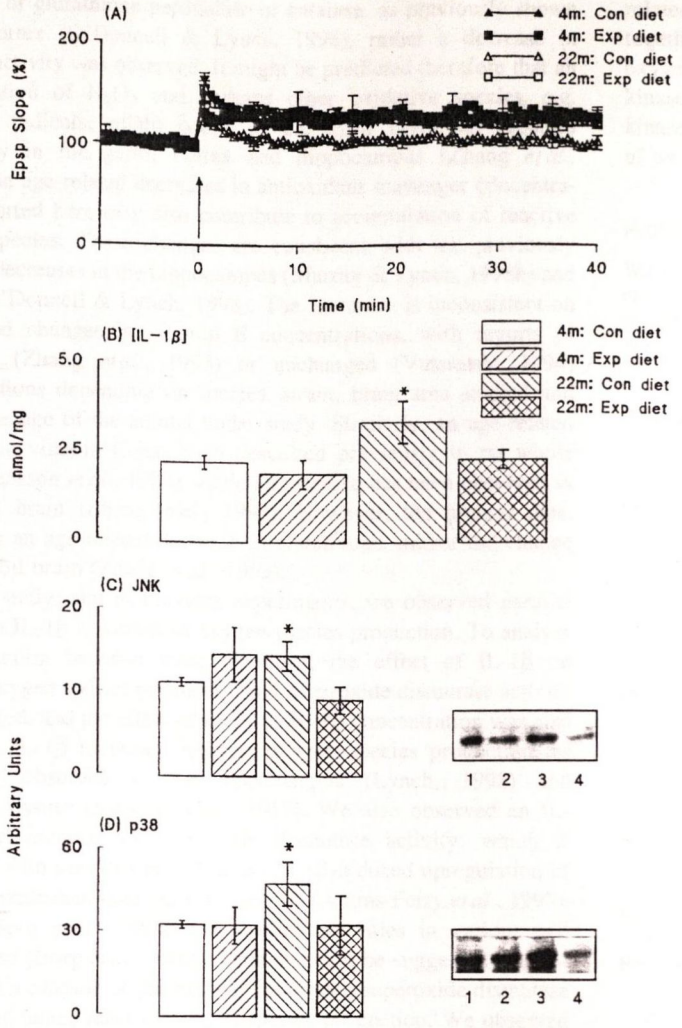


FIG. 6. LTP was impaired in aged rats fed on the control diet, but supplementation with a diet enriched in ω-3 fatty acids reversed this impairment. Diet did not affect LTP in young rats (A). IL-1β concentration was significantly enhanced in hippocampal tissue prepared from aged rats fed on the control diet compared with young rats fed on either diet, or aged rats fed on the experimental diet (B; $P<0.001$; Student's *t*-test for independent means). Activities of JNK (C) and p38 (D) were enhanced in hippocampal tissue prepared from aged rats fed on the control diet (lane 3) compared with young rats fed on the control diet (lane 1; $P<0.05$; Student's *t*-test for independent means); dietary manipulation reversed this aged-related increase (lane 4), while it increased variability in enzyme activities in hippocampal tissue prepared from young rats (lane 2).

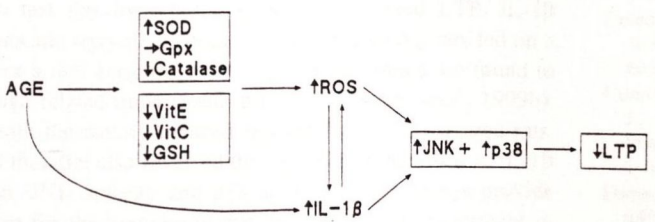


FIG. 7. Scheme suggesting cascade of events leading to the age-related impairment in LTP. Age is associated with (a) compromises in enzymatic and non-enzymatic antioxidant defences, and (b) an increase in IL-1β concentration. The compromise in antioxidant defence strategies leads to an increase in reactive oxygen species production, a change which might also result from increased IL-1β concentration. The evidence suggests a close coupling, and possibly positive feedback, between reactive oxygen species production and increased IL-1β concentration. Consequences of these changes include increased activity of JNK and p38, which may contribute to the impairment in LTP observed in aged rats.

JNK and p38 was directly assessed in hippocampal tissue and i which increased activity was described with age. In contrast to th increase in activity of JNK and p38, a decrease in activity of ERK ha been shown to accompany the age-related impairment in LTI (McGahon *et al.*, 1999a,b). This is consistent with the observatio that the MAP kinase inhibitor, PD98059, blocked LTP in CA (English & Sweatt, 1997) and dentate gyrus (McGahon *et al.*, 1999a)

We observed that the early response to tetanization was decrease in aged, compared with young, rats. Although we have observed thi effect previously using this strain of rats and under the sam experimental conditions (McGahon *et al.*, 1997; Murray & Lynch 1998a), others have reported no change in induction of LTP (e.g Barnes, 1979; deToledo-Morrell & Morrell, 1985). Although in al cases LTP in the dentate gyrus was studied, different experimenta conditions were employed, e.g. stimulating protocols, different ra strains and use of the unanaesthetized versus anaesthetized rats. I might be argued that the stimulating protocol used in this study wa less robust that those in the other studies, however, we observed tha these conditions induced saturated LTP in young rats (Williams *et al.* 1989) and induced LTP in aged rats fed on diets enriched in fatty acids, as shown here and elsewhere (McGahon *et al.*, 1997, 1999b) while these diets did not enhance LTP in young rats.

The data indicate that IL-1β concentration was significantly increased in tissue prepared from the hippocampus of aged rat compared with young rats, confirming our previously reporte findings (Murray & Lynch, 1998a,b; Lynch, 1998), and that IL-1β enhanced activity of both JNK and p38 *in vitro*. Modulation of thes kinases in neuronal tissue has not been investigated previously, but stimulatory effect of IL-1β on JNK activity has been reported i human glomerular mesangial cells (Uciechowski *et al.*, 1996) stromal cells (Rizzo & Carlo-Stella, 1996) and fibroblasts (Derijar *et al.*, 1994), while a stimulatory effect on p38 has been reported i human fibroblast-like synoviocytes (Miyazawa *et al.*, 1998). Chines hamster CCl39 cells (Guay *et al.*, 1997) and monocytic cells (Ha *et al.*, 1994; Raingeaud *et al.*, 1995). Given these observations and th present findings, it might reasonably be proposed that the age-relate increase in IL-1β concentration in the hippocampus stimulate activity of JNK and p38.

We also observed an age-related increase in reactive oxyge species production, which confirms our previous observation (Murray & Lynch, 1998a,b; Lynch, 1998), and, that like IL-1β H_2O_2 stimulated activity of both JNK and p38. To our knowledge thi is the first indication of such an effect in neuronal tissue, thoug stimulation of stress-activated protein kinases by oxidative stress ha been reported in other cell types previously (Schieven & Ledbette 1994; Tournier *et al.*, 1997). These findings are consistent with th hypothesis that the age-related increase in activities of JNK and p3 might result from the endogenous increases in IL-1β and/or reactiv oxygen species production.

We have previously coupled the age-related impairment in LT with increased IL-1β concentration (Murray & Lynch, 1998a,b), an have suggested that this may be a consequence of increased reactiv oxygen species production. The present findings suggest that one the consequences of increased endogenous IL-1β might be a increase in activity of JNK and p38, which in turn leads to inhibitio of LTP. Such a role for p38 is consistent with the observation that th p38 inhibitor, SB203580, overcomes the inhibitory effect of IL-1β LTP in the dentate gyrus *in vitro* (Coogan *et al.*, 1997).

The underlying cause of the age-related increase in reactive oxyge species production may result from the observed increase in activit of superoxide dismutase, an effect previously reported in the corte (O'Donnell & Lynch, 1998). Parallel increases were not observed

tivities of glutathione peroxidase or catalase, as previously shown the cortex (O'Donnell & Lynch, 1998), rather a decrease in talase activity was observed. It might be predicted therefore that an cumulation of H_2O_2 and perhaps other oxidative species, e.g. droxyl radicals, might occur in the aged brain, as reported eviously in the gerbil cortex and hippocampus (Zhang *et al.*, 93). The age-related decreases in antioxidant scavenger concentrations reported here may also contribute to accumulation of reactive ygen species. These changes are consistent with the previously ported decreases in the hippocampus (Murray & Lynch, 1998b) and rtex (O'Donnell & Lynch, 1998). The literature is inconsistent on e-related changes in vitamin E concentrations, with reports of creased (Zhang *et al.*, 1993) or unchanged (Vatassery, 1994) ncentrations depending on species, strain, brain area studied and e precise age of the animal under study. Similarly, an age-related crease in vitamin C has been described previously in rat whole ain (Svensson *et al.*, 1993), while an increase has been observed in e gerbil brain (Zhang *et al.*, 1993). However, the present data, scribing an age-related decrease in glutathione, mirror the change the gerbil brain (Zhang *et al.*, 1993).

In this study, and in previous experiments, we observed parallel anges in IL-1β and reactive oxygen species production. To analyse e interaction between these measures, the effect of IL-1β on active oxygen species production and superoxide dismutase activity as assessed, and the effect of H_2O_2 on IL-1β concentration was also easured. IL-1β increased reactive oxygen species production, as eviously observed in the hippocampus (Lynch, 1998) and eripheral tissues (Sumoski *et al.*, 1989). We also observed an IL-β-induced increase in superoxide dismutase activity, which is nsistent with previous reports of an IL-1β-induced upregulation of n-superoxide dismutase gene expression (Antras-Ferry *et al.*, 1997), RNA (Borg *et al.*, 1992) and enzyme activities in various cell reparations (Borg *et al.*, 1992). Thus, it might be suggested that IL-β triggers a cascade of reactions by increasing superoxide dismutase tivity and hence reactive oxygen species production. We observed at H_2O_2 increased IL-1β concentration, suggesting that a positive edback loop exists in which increased IL-1β stimulates increased active oxygen species production, while increased reactive oxygen ecies production in turn increases IL-1β. Analysis of this teraction requires further study, but it appears that when an age-lated increase in IL-1β concentration or reactive oxygen species oduction reaches a certain point, a positive feedback loop is imulated, which initiates a cascade of detrimental changes in euronal tissue leading to neurodegenerative changes.

Our proposal is that the age-related increase in IL-1β concentration creases activities of JNK and p38, and therefore negatively impacts n LTP. To test this hypothesis further we assessed LTP, IL-1β ncentrations and enzyme activities in aged and young rats fed on a ntrol diet or a diet enriched in ω-3 fatty acids, which we found to verse the age-related impairment in LTP (McGahon *et al.*, 1999b). n parallel with the dietary-induced restoration of LTP in aged rats, e observed that diet also reversed the age-related increases in IL-1β ncentration, JNK activity and p38 activity. The findings provide rong support for the hypothesis that the age-related impairment in TP is a consequence of increased IL-1β concentration, which may ct by enhancing activities of stress-activated kinases.

On the basis of the data presented here we present a scheme which utlines a proposed sequence of events leading to an age-related mpairment in LTP (see Fig. 7). The evidence suggests that age is ssociated with a compromise in antioxidant defences in the ippocampus and that this leads to an increase in reactive oxygen ecies production. This may be amplified by, or may induce, an age-related increase in IL-1β concentration in the hippocampus, and together the increased IL-1β concentration and increased reactive oxygen species production stimulated the stress-activated protein kinases, JNK and p38. It is proposed that increased activity of these kinases leads to impairment in neuronal function, one manifestation of which is an impairment in LTP in aged rats.

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Abbreviations

DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescein diacetate; DMSO, dimethylsulphoxide; DTC, dinitrophenylhydrazine/thiourea/copper; EPSP, excitatory postsynaptic potential; ERK, extracellular signal-regulated protein kinase; HPLC, high-performance liquid chromatography; IL-1β, interleukin-1β; LTP, long-term potentiation; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein kinase; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid.

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Short communication

Neuropeptide Y inhibits glutamate release and long-term potentiation in rat dentate gyrus

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stract

The effect of intracerebroventricular injection of neuropeptide Y (NPY) was assessed on LTP in dentate gyrus. We report that NPY nuated LTP and inhibited KCl-induced glutamate release in synaptosomes prepared from dentate gyrus. Activity of the stress-activated ase, c-Jun NH2-terminal kinase (JNK) in synaptosomes was increased by incubation with NPY or following intracerebroventricular ction. Activation of JNK might underlie the inhibitory effect of NPY on LTP. © 1999 Elsevier Science B.V. All rights reserved.

words: Long-term potentiation; Neuropeptide Y; c-Jun-activated protein kinase; Dentate gyrus; Glutamate release

Neuropeptide Y (NPY) is a 36 amino acid neuromodu- r secreted by neurons of the peripheral and central vous systems. It has been implicated in the control of ny physiological processes, including neuroendocrine ction [1] and the stress response [5]. It affects memory cessing, with evidence of improved performance in ne [13] but impairment in other [6] tasks.

NPY-immunoreactivity is concentrated in the hip- campus [10]. Five receptor subtypes have been identified] with high expression of Y_1 receptors in dentate gyrus d Y_2 receptors in other hippocampal regions [11]. Con- ent with the high density of Y_2 receptors in hippocam- s is the observation that this receptor mediates the ibitory effect of NPY on KCl-glutamate release in erfused hippocampal slices [12]. An NPY-induced inhi- ion of glutamate release was also observed in area CA1 which was considered to be a consequence of the ibitory action of NPY on calcium influx [8]. This posal is consistent with the inhibitory effect of NPY on and N-type calcium channels in pheochromocytoma ls [15]. In addition to the inhibitory effect of NPY on l transmission, an inhibitory effect in CA3 has also n described [18].

Evidence indicates that maintenance of LTP in dentate us is associated with increased glutamate release [2,4,16,17]. Since NPY exerts an inhibitory effect on glutamate release in area CA1, it was considered that a similar effect may occur in dentate gyrus, therefore, the effect of NPY was assessed on LTP in perforant path granule cell synapses. We report that NPY inhibited LTP and glutamate release and the evidence presented supports the idea that this is a consequence of NPY-induced activation of the stress-activated, mitogen-activated protein kinase, c-Jun NH2-terminal kinase (JNK).

Urethane-anaesthetized Wistar rats were injected intracerebroventricularly with NPY (10 ng/ml; 5 μl;) or saline (5 μl) and after 30 min, electrophysiological recording from the dorsal cell body region of the dentate gyrus, in response to perforant path stimulation, commenced. LTP was induced unilaterally as described [16]. Following a 40 min post-tetanic recording period, rats were killed and dentate gyri (untetanized and tetanized) were removed, frozen in Krebs solution containing 10% DMSO [14] and stored at −80°C.

Unstimulated and KCl-stimulated endogenous glutamate release was assessed in P_2 prepared from untetanized and tetanized dentate gyri or whole hippocampus by the filtration method [16]. The P_2 pellet was resuspended in oxygenated Krebs solution containing 2 mM $CaCl_2$, aliquotted onto Millipore filters (0.45 μm), rinsed and incubated in oxygenated Krebs solution at 37°C for 3 min in the presence or absence of 40 mM KCl to stimulate release. In some experiments, hippocampal synaptosomes were preincubated at 37°C for 15 min in the presence or

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absence of NPY (10 ng/ml; Sigma, UK). Glutamate concentrations in the filtrates were analysed as described [20]. Briefly, samples (50 μl) or glutamate standards (50 μl; 50 nM to 10 μM prepared in 100 mM Na_2HPO_4 buffer, pH 8.0) were added to glutaraldehyde-coated 96-well plates, incubated for 2 h at 37°C and washed in 100 mM Na_2HPO_4 buffer. Unreacted aldehydes were bound by ethanolamine (250 μl; 0.1 M in 100 mM Na_2HPO_4 buffer) and non specific binding was blocked by donkey serum (200 μl 3% in PBS-T). Antiglutamate antibody (raised in rabbit 100 μl; 1:5000 in PBS-T; Sigma, UK) was added, incubated overnight at 4°C, washed with PBS-T and reacted with secondary antibody (anti-rabbit horseradish peroxidase (HRP)-linked antibody (100 μl; 1:10,000 in PBS-T

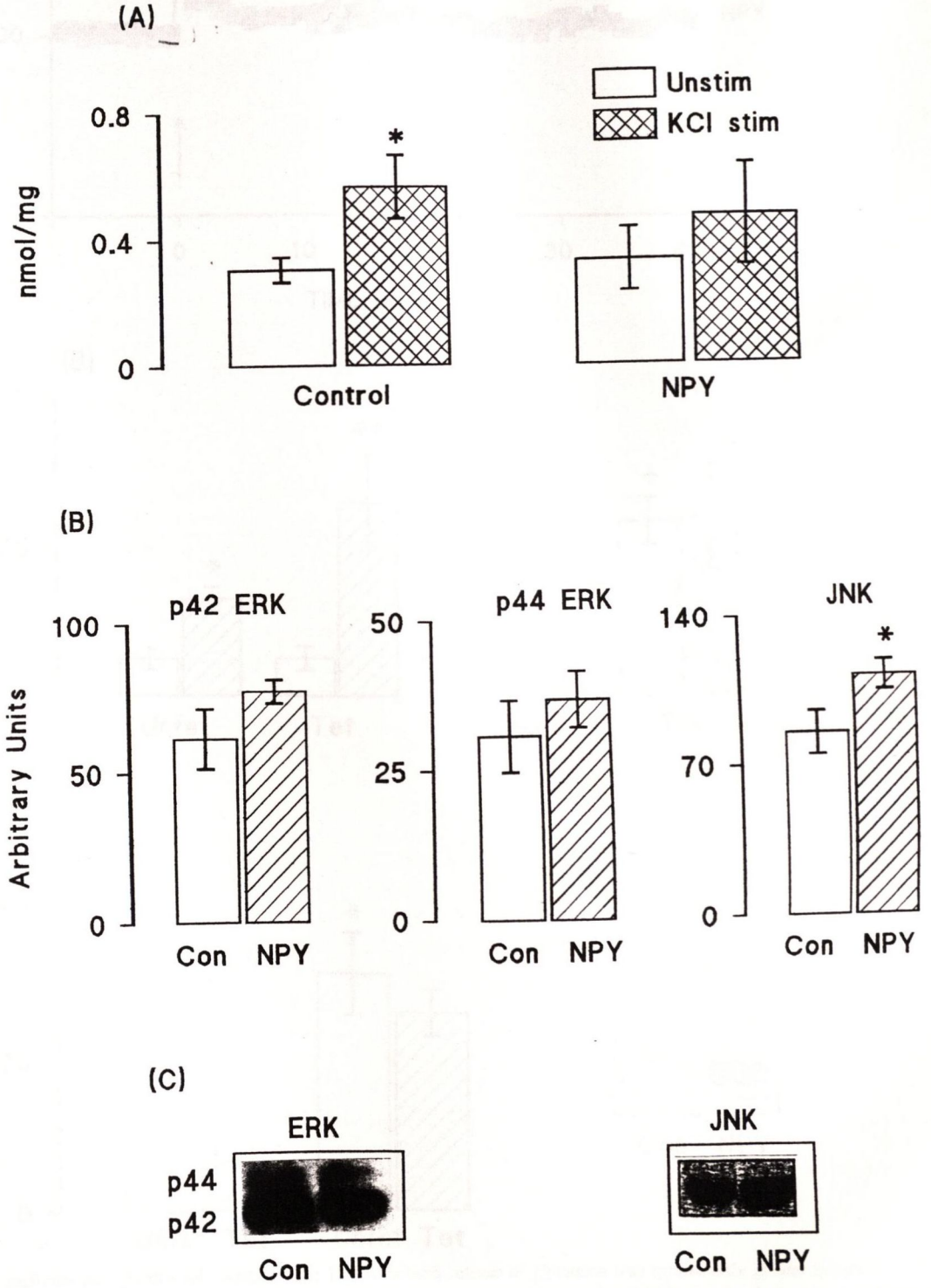

Fig. 1. NPY (10 ng/ml) significantly inhibited KCl-stimulated glutamate release in synaptosomes prepared from whole hippocampus ($p < 0.$ ANOVA). NPY did not affect ERK activity, but enhanced JNK activity ($p < 0.05$; Student's t-test for paired values).

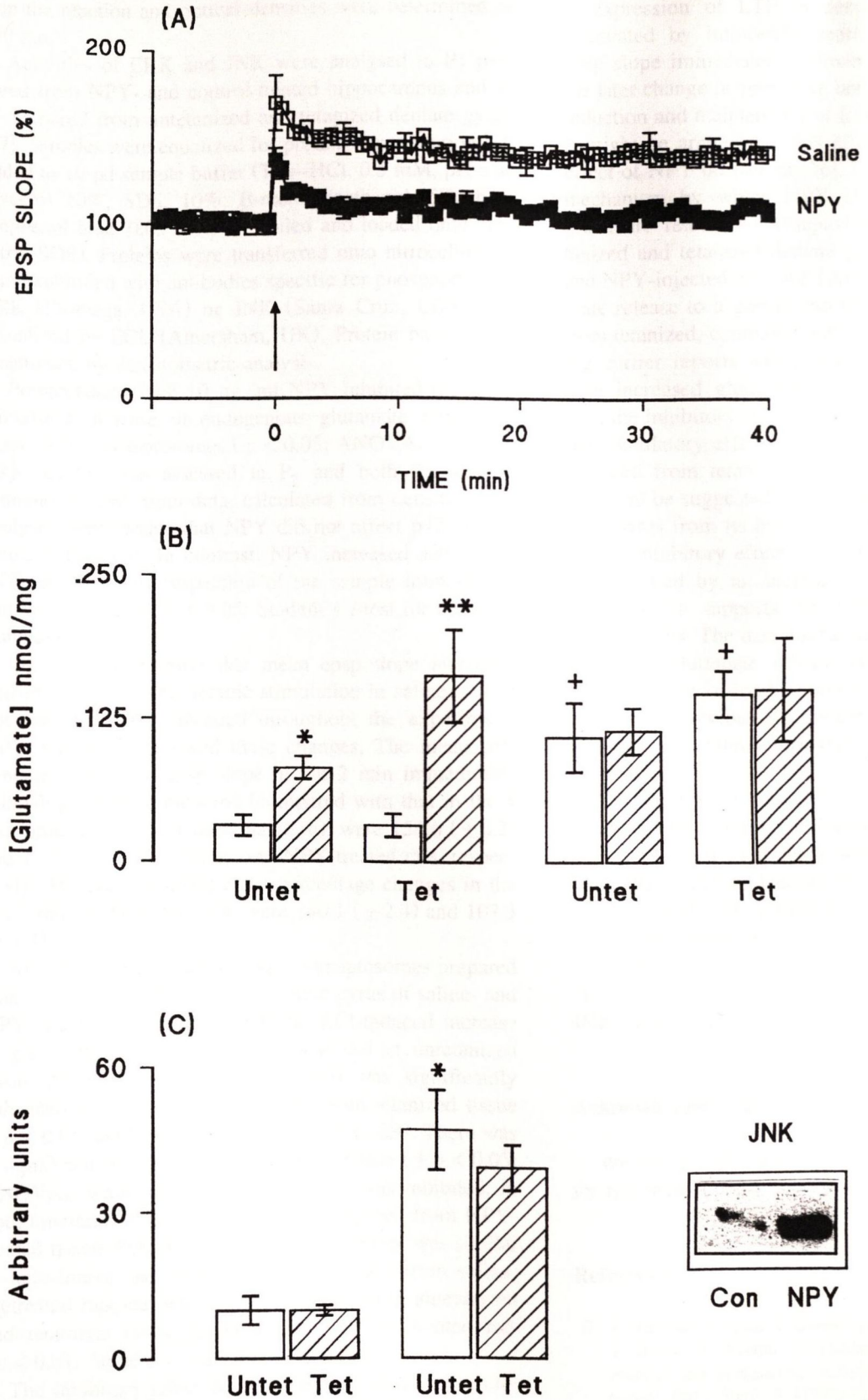

Fig. 2. NPY inhibited LTP in perforant path-granule cell synapses (A). KCl-stimulated release of glutamate was significantly greater in synaptosomes prepared from tetanized dentate gyrus, compared with untetanized dentate gyrus (B), but neuropeptide Y inhibited KCl-stimulated release of glutamate in both tetanized and untetanized preparations.

Amersham, UK). Samples were incubated with 3,3′,5,5′-tetramethylbenzidine, H_2SO_4 (4 M; 30 μl) was added to stop the reaction and optical densities were determined at 450 nm.

Activities of ERK and JNK were analysed in P_2 prepared from NPY- and control-treated hippocampus and in P_2 prepared from untetanized and tetanized dentate gyrus [17]. Samples were equalized for protein concentration [3], added to 10 μl sample buffer (Tris–HCl, 0.5 mM, pH 6.8; glycerol 10%; SDS, 10%; β-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v), boiled and loaded onto gels (10% SDS). Proteins were transferred onto nitrocellulose, immunoblotted with antibodies specific for phosphorylated ERK (Promega, USA) or JNK (Santa Cruz, USA) and visualized by ECL (Amersham, UK). Protein bands were quantitated by densitometric analysis.

Preincubation with 10 ng/ml NPY inhibited the KCl-stimulated increase in endogenous glutamate release in hippocampal synaptosomes ($p < 0.05$; ANOVA; Fig. 1A). ERK activity was assessed in P_2 and both the sample immunoblot and mean data, calculated from densitometric analysis, demonstrate that NPY did not affect p42 or p44 activity (Fig. 1B). In contrast, NPY increased activity of JNK, as shown by inspection of the sample immunoblot and the mean data ($p < 0.05$; Student's *t*-test for unpaired samples).

Fig. 2A demonstrates that mean epsp slope increased immediately following tetanic stimulation in saline-treated rats and remained enhanced throughout the experiment. NPY markedly attenuated these changes. The mean percentage changes in epsp slope in the 2 min immediately following tetanic stimulation (compared with that in the 5 min immediately prior to stimulation), were 158.6 (±6.2) and 124.0 (±3.8) in saline- and NPY-treated rats, respectively. The corresponding mean percentage changes in the last 5 min of the experiment were 140.1 (±2.4) and 107.3 (±1.8).

Analysis of glutamate release in synaptosomes prepared from untetanized and tetanized dentate gyrus of saline- and NPY-injected rats revealed that the KCl-induced increase in glutamate release which was observed in untetanized tissue prepared from saline-pretreated was significantly enhanced in synaptosomes prepared from tetanized tissue (*$p < 0.05$ and **$p < 0.01$; ANOVA; Fig. 2B). There was a significant increase in unstimulated release (+$p < 0.05$; ANOVA), while KCl-stimulated release was inhibited, in both untetanized and tetanized tissue prepared from NPY-treated tissue. Fig. 2C shows that JNK activity was similar in untetanized and tetanized tissue prepared from saline-pretreated rats, but was markedly enhanced in untetanized and tetanized tissue prepared from NPY-pretreated rats ($p < 0.01$; Student's *t*-test for independent means).

The inhibitory effect of NPY on KCl-stimulated glutamate release in hippocampal synaptosomes described here is in agreement with NPY-induced inhibitory effects observed previously in whole hippocampus [12], CA1 [7,8] and CA3 [18]. We observed that this effect was accompanied by an increase in activation of JNK, but not ERK.

Expression of LTP in dentate gyrus was markedly attenuated by intracerebroventricular injection of NPY epsp slope immediately following tetanic stimulation and the later change in epsp were both affected, suggesting that induction and maintenance of LTP were inhibited by NPY. It might be argued that this effect parallels the inhibitory effect of NPY on memory [6]. In an effort to establish the mechanism by which NPY inhibits LTP, we analysed glutamate release in synaptosomes prepared from untetanized and tetanized dentate gyrus obtained from saline and NPY-injected rats. We found that KCl increased glutamate release to a greater extent in synaptosomes prepared from tetanized, compared with untetanized, tissue confirming earlier reports which coupled LTP in dentate gyrus with increased glutamate release [2,4,16,17]. In parallel with the inhibitory effect of NPY on LTP, NPY attenuated the stimulatory effect of KCl on release in synaptosomes prepared from tetanized and untetanized tissue. It might therefore be suggested that the inhibitory effect of NPY on LTP arises from its inhibitory effect on glutamate release.

The inhibitory effect of NPY on LTP and release was accompanied by an increase in JNK activity in dentate gyrus, which supports the data obtained here in whole hippocampus. The data couple increased JNK activity with decreased glutamate release and compromised LTP in dentate gyrus; a combination of these effects have been observed in previous experiments in which LTP was inhibited by intracerebroventricular injection of interleukin-1β [21].

The question of how NPY activates JNK remains to be clarified. One possible mechanism might result from NPY interaction with G-proteins and subsequent activation of intracellular events leading to stimulation of JNK since NPY receptors are G-protein-coupled [19] and JNK stimulation can result from subsequent activation of cellular cascades [9]. Confirmation of our proposal that NPY-induced inhibition of LTP and release relies on activation of JNK awaits availability of a JNK inhibitor.

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